Complex interactions in the life cycle of a simple parasite shape the evolution of virulence

Luís M. Silva [1 *]

Jacob C. Koella [1]

[1] Institute of Biology, University of Neuchâtel, Rue Emilie-Argand 11, 2000 Neuchâtel, Switzerland

[*] Corresponding author: luis.macedo@unine.ch

ORCID

LMS: 0000-0002-8203-4006

Keywords: pathogen evolution, virulence, transmission, exploitation, microsporidia

Significance statement

In evolutionary medicine, we commonly refer to the severity or outcome of a parasitic infection as the parasite's virulence. The evolution of virulence is shaped by the properties of both the host and parasite, such as the strength of the host's immune response or the production of toxins by the parasite. A fundamental property of the parasite is the time at which it is transmitted from one host to the next. Here, using the mosquito *Anopheles gambiae* and the microsporidian parasite *Vavraia culicis*, we demonstrate that time to transmission directly affects the evolution of virulence and, therefore, the infection outcome in the new host. Our study highlights the importance of time to transmission in epidemiological models and decision-making in vector control strategies.

Abstract

Evolutionary expectations about the virulence of parasites (i.e. the parasite-induced mortality rate of the host) generally describe parasites which grow and are transmitted at the same time. Many parasites, however, go through an incubation period, that is they must grow before they can be transmitted. Moreover, the parasite's incubation period and virulence are linked through its growth rate. We here suggest that such interactions make it difficult to predict how virulence will evolve. We illustrate our ideas with a parasite with a simple cycle, the microsporidian *Vavraia culicis*, which infects the mosquito *Anopheles gambiae.* We selected the parasite for six generations to kill their host and be transmitted early or late. Selecting for late transmission led to a stronger increasing its exploitation (i.e., cost due to growth), but not pathogenicity per parasite (i.e., cost independent of growth), than selection for early transmission. This was associated with higher virulence, and a shorter parasite life cycle, with the latter having a larger and more rapid production of the infective spores. In response, the host shortened its life cycle and shifted to early fecundity when infected with late-selected spores. Altogether, the results of this study reinforce the need for a reviewed and unified virulence theory and raise important questions for epidemiological and vector control strategies.

1. Introduction

Virulence is generally defined as the degree to which a parasite reduces the fitness of its host[1]. It is the consequence of complex interactions between host and parasite strategies[2]. On one hand, the host influences it through its ability to clear the parasite (its resistance) or to limit its effects and associated costs (its tolerance)[3,4]. On the other hand, the parasite influences virulence with processes that are linked to using its host's resources and growing (exploitation) or that damage the host independently of the parasite's growth, such as toxins (per parasite pathogenicity)[2,5,6].

Despite this complexity, most ideas about the evolution of virulence are based on simple ideas: that virulence is governed by the parasite and that its evolution is constrained by its trade-off with the rate of transmission. Thus, to maximise a parasite's total transmission (that is, the rate of transmission times the period of infection), evolutionary pressure should favour a higher rate of transmission until the advantage of faster transmission is balanced by a shorter infection period due to greater virulence. Therefore, evolution should lead to an intermediate level of virulence[7,8]. While this and variations of this idea have been extensively discussed and studied for the last 50 years[7–9], empirical studies have given mixed support[10,11]. This is partly due to an overly simplistic description of transmission, one of the key parameters in infection. Some variations of transmission have been considered, and indeed whether transmission is vertical or horizontal[12] or whether a parasite is directly transmitted, has environmental stages or is vector-borne[13] strongly affects predictions about the evolution of virulence. However, a strongly overlooked factor is the timing of transmission, that is the time it takes for the parasite to grow, develop, and potentially evolve within its host before being transmitted.

According to the trade-off model earlier transmission should lead to higher virulence[14], though details of the timing of transmission and pathogenicity can lead to more complex predictions. One study that measured the effect of time to transmission was performed by Cooper and colleagues on nuclear polyhedrosis virus (NPV) infections in the moth *Lymantria dispar*[15]. They selected the virus for early or late transmission and tracked over generations the virulence and viral growth at two time points of the moth development. Selection for early transmission led to greater virulence but lower viral burden than selection for late transmission. While this result appears to agree with the virulence-transmission trade-off theory, it is difficult to interpret, for the experiment selected not only for early or late transmission but also inadvertently for rapid growth. This situation is made more complex by the fact that a longer time within the host before transmission might provide an opportunity for competing parasites to evolve[16,17]. Such competition is thought to be responsible for the evolution of higher virulence of the microsporidian *Glugoides intestinalis* when its host, *Daphnia magna*, is allowed to live longer[18]. Finally, part of the parasite's impact may be due to the phenotypic responses of the host, which are governed by trade-offs between early and late reproduction.

Here, we aimed to test if selection of the parasite's time to transmission impacts its virulence and if the evolution of virulence is translated into changes in infection dynamics due to parasite evolution

and shifts in host response. Then, we aimed to evaluate how the results fit within the modern virulence decomposition framework.

To accomplish this, we used the mosquito *Anopheles gambiae* and its microsporidian parasite *Vavraia culicis*, which is a common generalist parasite of mosquitoes with low costs to the host[19,20] and whose life cycle allows us to easily control and manipulate both time-to and mode-of transmission. With this approach, we hoped to shed light on the role and importance of considering time to transmission in evolutionary and epidemiological studies. The latter is particularly relevant in vector-borne disease evolution where most vector-transmitted parasites require long-lived hosts or vectors to complete their life cycle and therefore will carry co-infections with late transmission.

2. Materials and methods

2.1 Experimental model

We used the microsporidian parasite *Vavraia culicis floridensis*, provided by James Becnel (USDA, Gainesville, FL) and the Kisumu strain of the mosquito host *Anopheles gambiae (s.s)*. Mosquitoes were reared under standard laboratory conditions (26 ± 1ºC, 70 ± 5% relative humidity and 12 h light/dark). *V. culicis* is a generalist, obligatory, intracellular parasite that infects mosquito epithelial gut cells. Mosquitoes are exposed to the microsporidian during the larval aquatic stage, where they ingest spores in the water and food. After replication within the larvae, they start producing spores from within the adult gut cells, re-infecting new cells and propagating the cycle until they kill the host. Spores are transmitted when infected individuals die in water or from mother to offspring by adhering to the surface of the eggs. Before starting the experiment, *V. culicis* was kept in large numbers by alternatingly infecting Aedes *aegypti* and *An. gambiae* populations, to ensure it remains a generalist parasite. The parasite was allowed to develop within these populations until host death when *V. culicis* spores were retrieved for later infections.

2.2 Experimental evolution and rearing

A starting population of 300 freshly hatched *An. gambiae* larvae were grown in groups of 50 per petri dish (120 mm diameter x 17 mm) containing 80 mL of distilled water. Larvae were fed with daily Tetramin Baby fish food according to their age: 0.04, 0.06, 0.08, 0.16, 0.32 and 0.6 mg/larva for 0, 1, 2, 3, 4 and 5 days or older, respectively. On the second day of development, individuals were exposed to 10,000 spores of *V. culicis* per larva. Upon pupation, individuals were transferred to cages (21 x 21 x 21 cm) and left to emerge into adults. Freshly prepared cotton pads with 6% sucrose solution were provided throughout their adult life. At adulthood, two selection regimes were generated (Fig. 1a): i) Early transmission, where the spores from the first 50 females to die were retrieved and used to infect the following generation of the same regime; ii) and late transmission, where the spores from the last 50 females to die were used. Each regime consisted of five replicate populations and was selected for

six generations prior to assessing differences due to selection at F7. To ensure transmission only happened through parasite killing, dead individuals were removed shortly after death, and female mosquitoes were never blood-fed and therefore unable to reproduce. The retrieved dead females were kept in 2mL microcentrifuge tubes with 1 mL of distilled water at 4ºC for no longer than 40 days, to maintain the viability of the spores. At the end of each generation, we added a stainless-steel bead (Ø 5 mm) to each of the tubes and homogenized the samples using a Qiagen TissueLyser LT at a frequency of 30 Hz for one minute. The spore concentration was then estimated by counting spores in a haemocytometer under a phase-contrast microscope (400x magnification) and the dose for the next generation was adjusted to 10,000 spores/larva. We only selected the parasite, and therefore, we used mosquitoes from the stock population to start every generation.

2.3 Response to selection

After six generations of selection, we measured the effect of parasite selection on a set of host traits: larvae and pupae mortality, time to pupation, body size, fecundity, longevity and infection dynamics for each of the *V. culicis* selection regimes and replicates. We included the "stock" *V. culicis* population as a regime as our baseline reference. All the common garden experiments were performed in a similar design to the selection protocol unless said otherwise. Both larvae and pupae mortality, as well as time to pupation, were assessed every 24 ± 1 h. To assess fecundity, adults were allowed to mate in a cage (21 x 21 x 21 cm) for the first seven days of adulthood. The males were removed on the seventh day. Two days prior to the assay day, females were allowed to blood feed for five minutes on Tiago G. Zeferino's arm. Two days later, 100 females from each treatment and replicate were individualized in cups (5 x 5 x 10 cm) with Whatmann filter paper (110mm) and water and allowed to lay eggs for 48 hours. Afterwards, all females were killed, and the number of eggs counted. Body size at adult emergence was measured using wing length at day 5 as a proxy [12]. Longevity was tracked in adult females, which were kept in individual cups (5 x 5 x 10 cm) with access to fresh sugar solutions and a humid filter paper. Infection dynamics was followed by sacrificing alive females at day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 18 post adult emergence and then quantifying their spore load, as described in the previous section.

2.4 Statistical analysis

All the analyses were performed with R version 4.3.1 in RStudio version 3034.06.2+561. The following packages were used for visualizing and plotting the data: “ggplot2”[21] , "purrr"[22], "scales"[23], "dplyr"[24] and "tidyr"[25], as well as created with BioRender.com and edited in Microsoft PowerPoint 16.16.27. Statistical analysis and their diagnosis were conducted using: “DHARMa”[26], “car”[27], “survival”[28], "lme4"[29], "muhaz"[30], "emmeans"[31] and "multcomp"[32] .

Longevity was compared across regimes using a cox proportional hazard with the regime as the explanatory variable and the replicate as a random factor. Then, we generated a survival curve for each replicate of each regime and extracted its maximum hazard, as in[6], our proxy for virulence. Differences in virulence were assessed through a linear model with the natural log of the maximum hazard as a dependent variable, the regime as the explanatory variable and the replicate as a random factor.

Concerning the cost of infection in fecundity, we first tested if the proportion of non-egglaying females was the same across all regimes and days for each replicate. For this, we ran a generalized linear model with a binomial structure and both regime and day post-adult emergence as fixed factors, as well as their interaction, and replicate as a random factor. There was no effect of any of the treatments and therefore, only considering females who had laid eggs, we calculated the percentual cost of infection by subtracting the mean uninfected fecundity from each infected individual fecundity, for day 10 and 15 of adulthood, and dividing the resulting value by the infected individual fecundity and multiplying it by 100. Then, we assessed differences due to regime and/or days post-adult emergence using a linear model with replicate as a random factor.

Larvae and pupae mortality were measured as the proportion of dead individuals, for each of the stages. A generalized linear model with a binomial error structure, and the replicate as a random factor, was used to determine the effect of regime on each of the developmental stage mortality. Individuals pupated from day 6 to 11 post eclosion, but approximately 77%, 79% and 82% of them pupated on day eight or nine, for Early, Late and Stock, respectively. Therefore, we split individuals into two categories, the ones that pupated until day eight and the ones that pupated from day nine onwards and used a generalized linear model with binomial distribution to measure differences in early pupation. As before, the regime was considered an explanatory variable and the replicate as a random factor.

We defined spore rate as the proportion of females with detectable spores per regime for each of the first ten days of adulthood. Our detection threshold is estimated to be 100 spores. Mosquitoes with detectable spores were then used to estimate spore dynamics from day one to 18 of adulthood. The spore rate was analysed using a generalized linear model with a binomial distribution, while for spore dynamics we used a linear model to assess differences in the log-transformed number of spores. In both models, we had regime and day, and their interaction, as explanatory factors and the replicate as a random factor.

To decompose virulence, we used the maximum hazard from the longevity curves and (since the average maximum hazard day was 20) the spore density on days 15 and 18. Spore density was calculated as the individual spore load at days 15 and 18 adjusted by individual wing length. Virulence decomposition analysis was done as in [6]. In summary, exploitation was measured as the natural log-transformed mean spore density from days 15 and 18 for a given regime and replicate, while per parasite pathogenicity was given as the slope of the relationship between natural log-

transformed maximum hazard and (i.e., virulence) and exploitation. Lastly, post-hoc multiple comparisons were performed with "emmeans", using the default Tukey adjustment, across the different models.

3. Results

The selection regime affected the host's longevity, with the individuals infected with spores selected for late transmission living less long than or with unselected spores ($\chi2 = 138.82$, $df = 2$, $p < 0.001$; Fig. 1b). The comparison of the maximum hazard from the replicates of each regime gave a similar result, with the infection from late-selected spores leading to an increase in maximum hazard and therefore virulence ($\chi2 = 13.239$, $df = 1$, $p < 0.001$; Fig. 1c). See SI Appendix Table S1 for further details on these analyses.

The cost of infection in fecundity was strongly affected by the selection regime ($df = 2$, F= 5.914, $p = 0.003$; Fig. 1d), with parasites selected for early or late transmission reducing fecundity less than the unselected parasites. It was also affected by day ($df = 1$, F= 20.280, $p < 0.001$; Fig. 1d), with the cost increasing from day 10 to 15. Although there was no significant effect of interaction between the selection regime and day, a multiple comparisons test was performed to measure differences between each combinatorial treatment. In particular, the fecundity of individuals infected with Late parasites on day 10 was decreased less than either that of individuals with Late parasites on day 15 ($df = 258$, t-ratio = 4.164, $p<0.001$) or unselected parasites on day 10 ($df = 258$, t-ratio = 3.533, $p<0.001$) or 15 ($df = 258$, t-ratio = 4.916, $p=0.006$). Further statistical details on SI Appendix Table S1.

Furthermore, spores selected for late transmission were more likely to kill larvae than unselected parasites ($z = 2.559$, $p = 0.028$; Fig. 2a), while selection for early transmission increased pupal mortality ($z = 2.520$, $p = 0.032$; Fig. 2b). Late transmission also reduced the time to pupation ($z = 3.709$, $p < 0.001$; Fig. 2c), but did not impact adult size ($\chi2 = 0.074$, $df = 2$, $p = 0.964$; Fig. 2d). Further details can be found in the SI Appendix Table S2.

The selection regime also impacted the infection parameters. Thus, the spore rate increased with time ($\chi2 = 114.375$, $df = 1$, $p < 0.001$), but this increase was strongest for parasites selected for late transmission and lowest for unselected parasites (interaction between selection regime and day: $\chi2 = 24.854$, $df = 2$, $p < 0.001$) (Fig. 3a). The number of spores was also affected by selection regime ($\chi2 = 41.857$, $df = 2$, $p < 0.001$), day ($\chi2 = 158.934$, $df = 1$, $p < 0.001$) and their interaction ($\chi2 = 10.993$, $df = 2$, $p = 0.004$). Thus, the average number of spores increased with the progression of infection, and did so most strongly if the parasites had been selected for late transmission (Fig. 3b). Additional details are in SI Appendix Table S3.

These changes in infection dynamics had consequences for the decomposition of virulence. Exploitation (i.e., cost due to parasite growth) was affected by the selection regime ($df = 2$, F = 55.466, $p < 0.001$), but per parasite pathogenicity was not ($df = 1$, F = 0.302, $p = 0.602$) (Fig. 4; See

SI Appendix Table S4 for statistical comparisons). The figure suggests that, although we did not detect a significant effect of selection for early transmission on virulence, it did increase the parasite's ability to exploit the host, though to a lesser degree than selected for late transmission. Considering *V. culicis* growth reaches a plateau around days 15 and 18 of adulthood (unpublished work), we also used spore density as a proxy of transmission and tested for a relationship between transmission and virulence in this infection model system. Using a linear model, we detected an effect of virulence (i.e., natural log of the maximum hazard) on transmission (i.e., natural log of mean spore density) ($df = 1$, $F = 31.534$, $p < 0.001$, Fig. 4c, statistical details on SI Appendix Table S4), providing evidence for a positive correlation between both in this system.

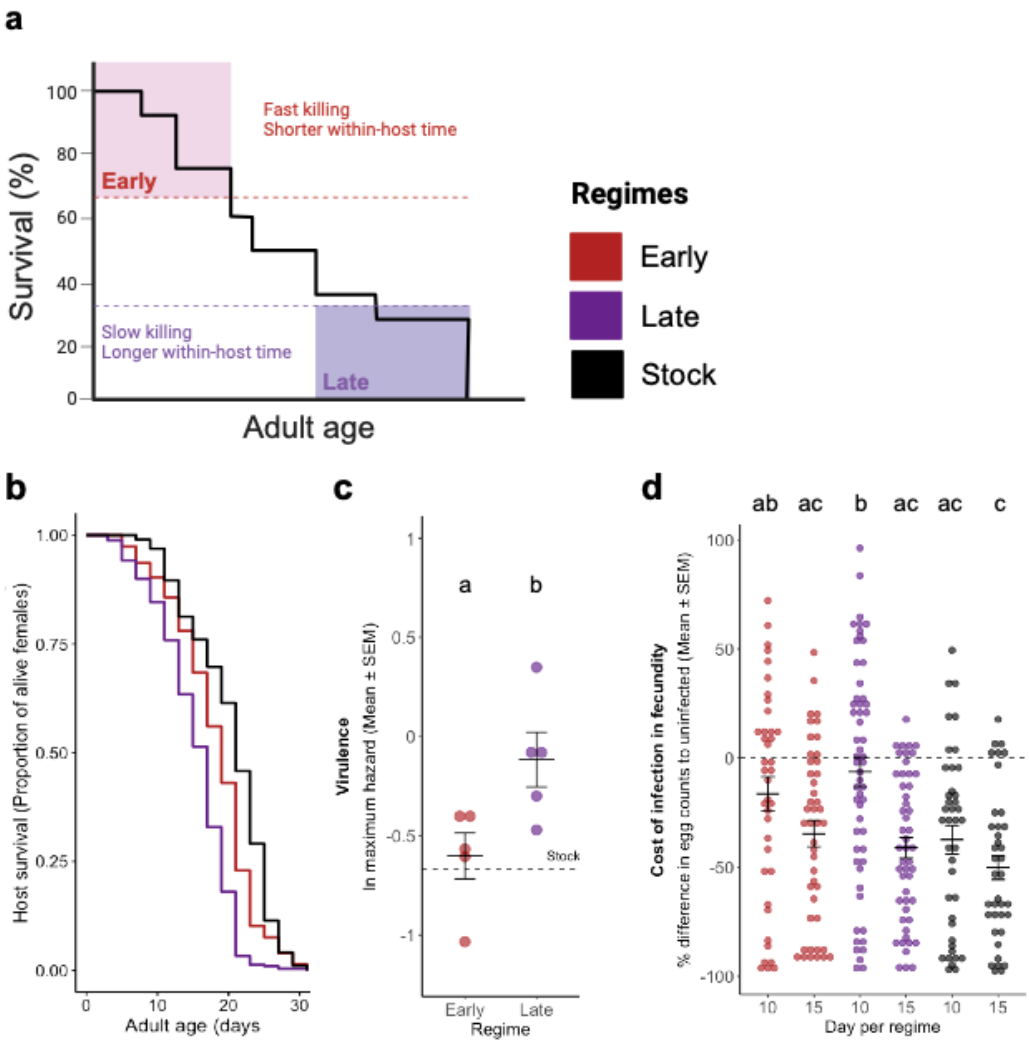

Figure 1. Longevity, fecundity and virulence evolution. (a) Experimental design, describing direct and indirect forms of parasite selection in this study. **(b)** Host survival after infection with evolved or reference parasites. Early and Late curves were generated with 487 and 519 females split throughout

five replicates. The stock was composed of 96 females. **(c)** Virulence, as the natural log of the maximum hazard for each of the replicates of each selection regime (n = 5). The dashed line represents the virulence of the parasite stock. See SI Appendix Table S1 for a complete statistical analysis. **(d)** Cost of infection in fecundity, as the percentage difference in egg numbers of infected mosquitoes comparatively to the uninfected ones, for days 10 and 15 post adult emergence. The dashed line corresponds to the reference fecundity or uninfected individuals. The sample size was the following: Early with 39 and 45, Late with 56 and 50, Stock with 40 and 38 for days 10 and 15, respectively.

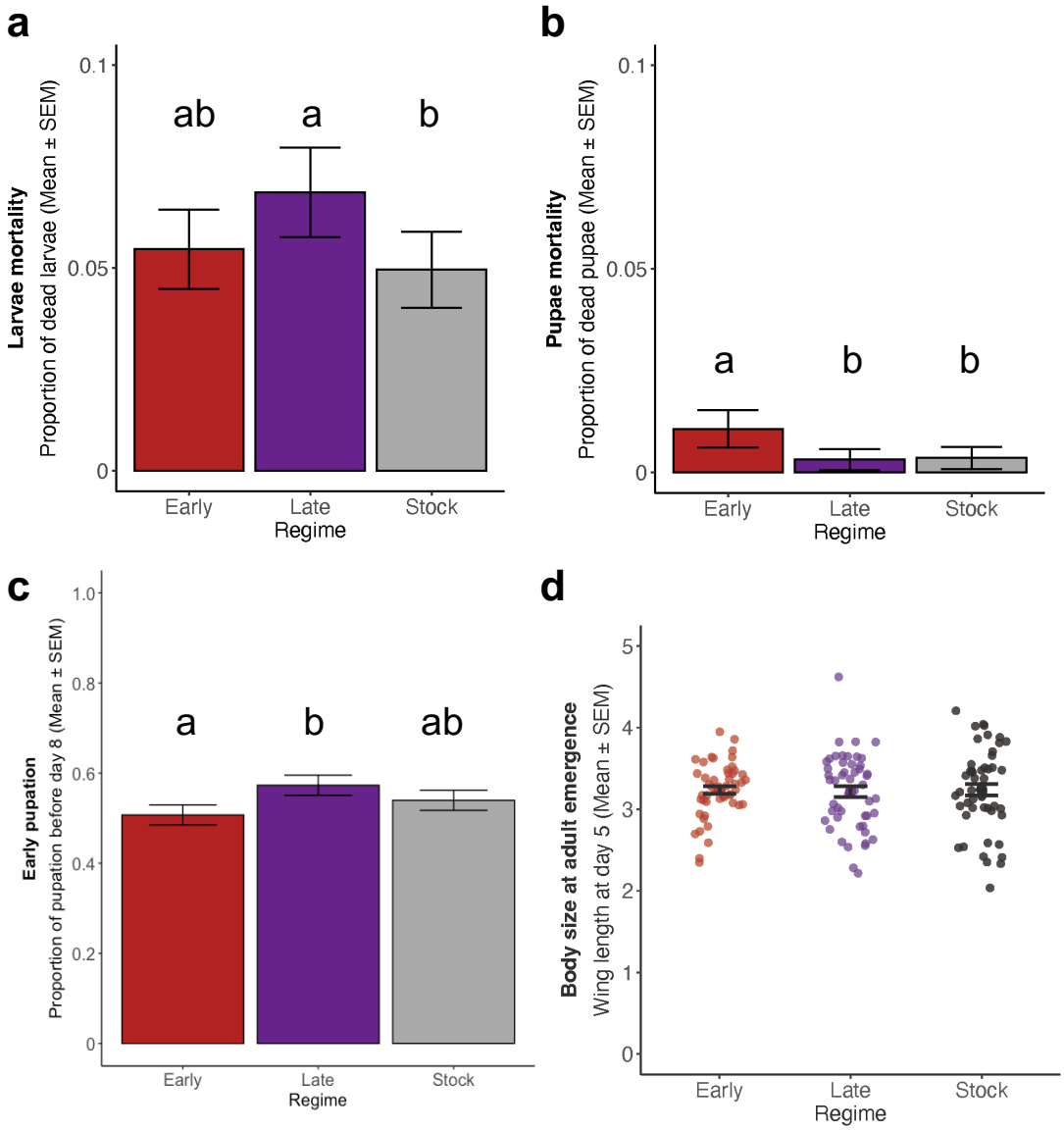

Figure 2. Host developmental traits. (a) Larvae mortality across regimes. The regimes were composed of 2068, 2011 and 2037 individuals for Early, Late and Stock. **(b)** Pupae mortality with each regime consisting of 1955, 1873 and 1936 in the same regime order. **(c)** Early pupation, as the proportion of individuals that pupated until day eight post eclosion. In the same regime order, the

sample size was 1934, 1867 and 1929 individuals. **(d)** Body size at adult emergence using wing length of five-day-old females as a proxy. Each regime consisted of 50 individuals. Different letters denote means that they are significantly different from one another. See SI Appendix Table S2 for complete statistical analysis.

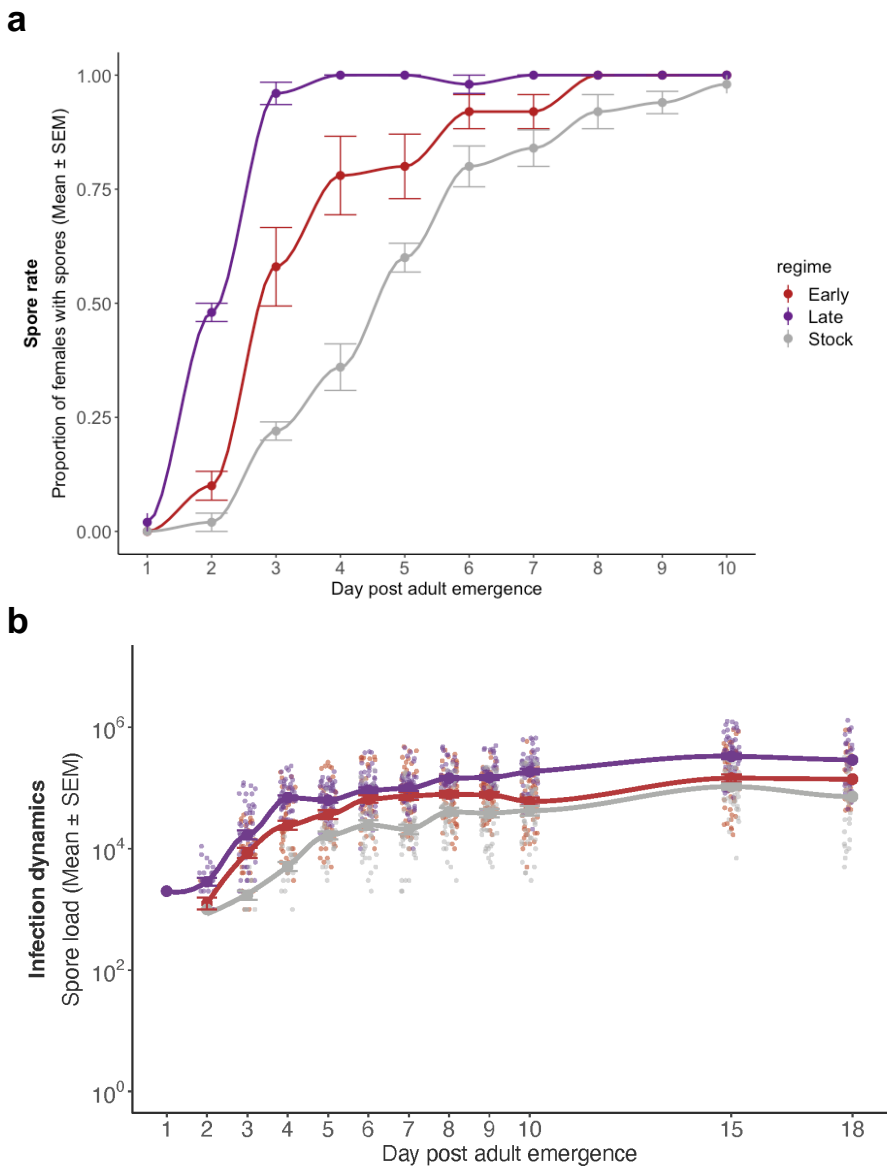

Figure 3. Infection dynamics. (a) Spore rate, as the daily proportion of females with detectable spores for each treatment during the first ten days of adulthood. **(b)** Spore dynamics, as the mean number of spores per female for each of the first ten days, as well as days 15 and 18 (i.e., chronic load). For each regime and time-point, 50 females were assayed. See SI Appendix Table S3 for complete statistical analysis.

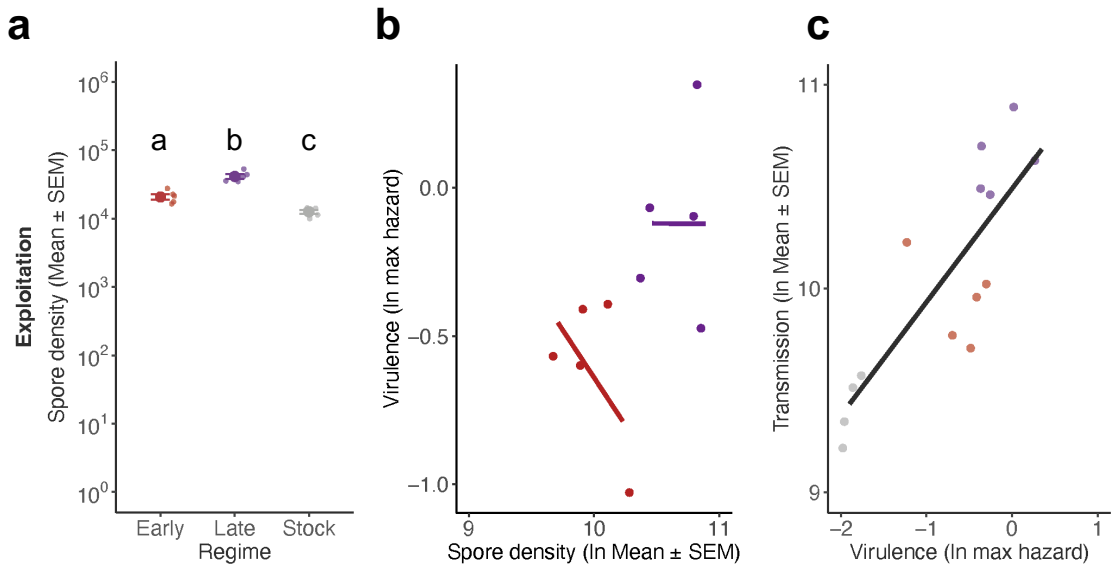

Figure 4. Virulence decomposition. (a) Exploitation was measured as the mean spore density at days 15 and 18 (i.e., spore load divided by wing size) for every replicate of each regime. **(b)** Per parasite pathogenicity, as the relationship between virulence and spore density. **(c)** Transmission-virulence relationship for this infection model. Given that in this infection model, the spore load is expected to decelerate and plateau in the measured chronic phase, the number of spores is not expected to increase and therefore provide a fair estimate of the total number of spores each of the regimes is able to transmit. There is a positive relationship between virulence and transmission ($df = 1$, $F = 31.534$, $p < 0.001$). Different letters denote means that they are significantly different from one another. See SI Appendix Table S4 for further details on the statistical analysis.

4. Discussion

We demonstrated that time to transmission affects the evolution of virulence. In particular, we showed that contrary to the predictions of a theoretical model[14], a longer time within the host (i.e., late transmission) selected for greater virulence than a shorter time (i.e., early transmission). Our results were also the opposite of a study on nuclear polyhedrosis virus (NPV) infections in the moth *Lymantria dispar*[15], in which selection of the virus for late transmission led to lower virulence (and higher viral burden) than selection for early transmission.

The difference in results emphasises the complexity of the evolution of virulence. In particular, it may result from the different levels at which selection operates on parasites: the competition among parasites within hosts and the transmission among hosts. Indeed, if selection operates at the level of transmission among hosts, as assumed by much of the theory of the evolution of virulence[7,8] and by Osnas and Dobson (2010), selection for early transmission would lead to a higher killing rate than

selection for late transmission. A main reason for our opposite result may be that in our experiment the main sources of selection operate within the host.

First, since we selected for early and late transmission by letting the parasite be transmitted from mosquitoes that died early or that lived a long time, we indirectly selected for shorter and longer periods of infection. A longer period of infection, in turn, increases the chance of mutation or the strength of density-dependence as the parasite load increases, and thus increases the possibility of competition among parasite genotypes[33], in a phenomenon described as clonal interference[16]. This selects for more transmissible or more rapidly replicating parasites in our study (Fig. 3 and 4) and others[16,33]. This result is experimentally supported by a recent study from Sheen and colleagues (2024), which suggested that in the absence of turnover rates in poultry markets, and longer host lifespan, virulence is no longer expected to maintain at intermediate levels but instead continuously increase as observed with circulating parasites such as H5N1[34]. We expect this phenomenon to happen not only in poultry markets with lower turnover but also in society and the wild. Longer infection gives the immune response more time to act, giving greater selective pressure for the parasite to evade immunity and, consequently, replicate more rapidly and be more virulent[35], thus hindering the fixation of genotypes with low virulence[36]. In addition, the host's immune response should increase at higher parasite burdens[6,37] Therefore, early-transmitted parasites, which have had little time to replicate, are subjected to less selective pressure from the host's immune response. Late-transmitted parasites, however, grow to a higher density and are subjected to a strong immune response, which then fades with the mosquito's age[38,39], letting the selected genotypes replicate rapidly. Overall, thus, in contrast to selection due to transmission among hosts, within-host processes are likely to select for high virulence of parasites that can replicate for a long time and that transmit late.

A second possibility is a consequence of the experimental restriction of the parasite's transmission route. Although *V. culicis* is not an obligate killer (see Materials and Methods section 2.1), the experimental design made it behave like one. Evolutionary models about obligate killers[11,40–42] rely on the assumption that the host is a limited resource so that the parasite's growth is density-dependent, and that the parasite's growth is decoupled from its transmission. This makes it possible for "selfish" genotypes to grow without counter-selection[40,43]. Both assumptions are likely to hold in our experiment. In particular, our protocol eliminated many costs of transmission. Thus, for example, since spores were sampled shortly after their production, transmission was independent of their long-term quality or infectivity, so any trade-off between the replication rate and quality of spores played no role in transmission[11,44,45]. Indeed, the positive correlation between chronic spore load (i.e., a proxy for transmission) and virulence (when results of all selection regimes and replicates are pooled) (Fig. 4c) corroborates this uncontrollable growth hypothesis. Several studies and models have supported this hypothesis[5,46,47].

Several other results are noteworthy. First, adaptation to either early or late transmission increased the parasite's prevalence of spores (Fig. 3a), growth (Fig. 3b) and exploitation of the host (Fig. 4a). This is most likely because the parasite switched from being maintained on two mosquito species as hosts to a parasite infecting only on *An. gambiae*. The parasite could thus specialise on a single host without being impeded by the cost of having to also perform on a second one[48,49]. However, selection did not affect per-parasite pathogenicity for any of the regimes (i.e., damage independent of parasite growth, Fig. 4b). This is likely due to the limited microsporidian genome[50] and, to our knowledge, lack of virulence factors on which selection can act.

Second, our selection regime had an impact on some aspects of the mosquitoes' development – late-selected parasites shortened larval development and increased larval mortality – but had no impact on adult body size (Fig. 2). An increase in the larval mortality might be an immediate cost of infection with late-selected spores. Spores that are more virulent and come with larger growth rates are likely to quickly extinct any susceptible host genotype in the population. Conversely, early-selected spores seem to not possess as high growth rates, and therefore, the cost in pupal mortality might reflect a cumulative cost of the increased parasite growth compared to stock-infected hosts.

Third, infection decreased fecundity, in particular later in life. This impact does not appear to be linked to the density of spores, for the average number of spores did not differ between the time points when fecundity was measured and infection by the unselected parasites gave the lowest fecundity despite having low spore loads (Fig. 3). Rather the pattern may reflect the host's response to infection. Indeed, as suggested by the life-history theory[51], the shorter the lifespan of infected mosquitoes was, the greater the early investment in fecundity, while late fecundity was low and similar in each selection regime.

Altogether, our study highlights the importance of considering the timing of transmission in studies of infection and virulence evolution. We also validated many of the concerns previously discussed in literature concerning the current virulence evolution model and reinforced the need for a modern unified theory of virulence. Although usefully simplistic, the current theory of virulence disregards a considerable portion of parasite strategies. Lastly, we believe our study has major consequences for epidemiology and vector-control strategies. For instance, we provide evidence that shortening of a vector life cycle might not affect population numbers, as treatments with shorter lifespan have a greater earlier investment in fecundity (Fig. 1d) and therefore might compensate for the shorter time to reproduce. Moreover, microsporidia have been shown to suppress *Plasmodium* infections in wild- and lab-grown mosquitoes[52,53], having potential as control agents for malaria[19,54]. Simultaneously, *Plasmodium* infection requires long-lived mosquitoes to complete its life cycle, which by extension selects for co-infection with late-transmitted parasites such as the one in this study. It is completely unknown if this has an impact on this tripartite interaction or if there are consequences for the *Plasmodium* life cycle and pathogenicity. Nevertheless, this system seems a unique model to test

microbe-driven vector-control mechanisms, while allowing to study of fundamental evolutionary questions regarding infection and microbe interactions.

Author contributions

LMS conceived the overall idea. JCK and LMS designed the experiments. LMS collected and analysed the data. LMS wrote the first draft of the manuscript. Both authors contributed critically to the drafts.

Acknowledgements

We thank Tiago G. Zeferino and Gwendoline Acerbi for their advice and technical support. LMS and the project were supported by SNF grant 310030_192786.

References

1. Read, A. F. The evolution of virulence. *Trends Microbiol.* **2**, 73–76 (1994).
2. Råberg, L. & Stjernman, M. The evolutionary ecology of infectious disease virulence. *Ecoimmunology* **548**, 78 (2012).
3. Råberg, L., Graham, A. L. & Read, A. F. Decomposing health: tolerance and resistance to parasites in animals. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 37–49 (2009).
4. Kutzer, M. A. M. & Armitage, S. A. O. Maximising fitness in the face of parasites: a review of host tolerance. *Zoology* **119**, 281–289 (2016).
5. Fraser, C., Hollingsworth, T. D., Chapman, R., de Wolf, F. & Hanage, W. P. Variation in HIV-1 set-point viral load: epidemiological analysis and an evolutionary hypothesis. *Proc. Natl. Acad. Sci.* **104**, 17441–17446 (2007).
6. Acuña-Hidalgo, B., Silva, L. M., Franz, M., Regoes, R. R. & Armitage, S. A. O. Decomposing virulence to understand bacterial clearance in persistent infections. *Nat. Commun.* **13**, 1–14 (2022).
7. Anderson, R. M. & May, R. M. Coevolution of hosts and parasites. *Parasitology* **85**, 411–426 (1982).
8. May, R. M. & Anderson, R. M. Epidemiology and genetics in the coevolution of parasites and hosts. *Proc. R. Soc. London. Ser. B. Biol. Sci.* **219**, 281–313 (1983).
9. Cressler, C. E., McLeod, D. V, Rozins, C., Van Den Hoogen, J. & Day, T. The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. *Parasitology* **143**, 915–930 (2016).

10. Alizon, S., Hurford, A., Mideo, N. & Van Baalen, M. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* **22**, 245–259 (2009).
11. Alizon, S. & Michalakis, Y. Adaptive virulence evolution: the good old fitness-based approach. *Trends Ecol. Evol.* **30**, 248–254 (2015).
12. Agnew, P. & Koella, J. C. Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proc. R. Soc. London. Ser. B Biol. Sci.* **264**, 9–15 (1997).
13. Day, T. Parasite transmission modes and the evolution of virulence. *Evolution (N. Y).* **55**, 2389–2400 (2001).
14. Osnas, E. E. & Dobson, A. P. Evolution of virulence when transmission occurs before disease. *Biol. Lett.* **6**, 505–508 (2010).
15. Cooper, V. S. *et al.* Timing of transmission and the evolution of virulence of an insect virus. *Proc. R. Soc. London. Ser. B Biol. Sci.* **269**, 1161–1165 (2002).
16. Strelkowa, N. & Lässig, M. Clonal interference in the evolution of influenza. *Genetics* **192**, 671–682 (2012).
17. Pan, G. *et al.* Invertebrate host responses to microsporidia infections. *Dev. Comp. Immunol.* **83**, 104–113 (2018).
18. Ebert, D. & Mangin, K. L. The influence of host demography on the evolution of virulence of a microsporidian gut parasite. *Evolution (N. Y).* **51**, 1828–1837 (1997).
19. Lorenz, L. M. & Koella, J. C. The microsporidian parasite Vavraia culicis as a potential late life–acting control agent of malaria. *Evol. Appl.* **4**, 783–790 (2011).
20. Lorenz, L. M. & Koella, J. C. Maternal environment shapes the life history and susceptibility to malaria of Anopheles gambiae mosquitoes. *Malar. J.* **10**, 1–8 (2011).
21. Wickham, H. *ggplot2: elegant graphics for data analysis*. (springer, 2016).
22. Henry, L. & Wickham, H. purrr: Functional Programming Tools. R package version 0.3. 4. (2020).
23. Wickham, H., Wickham, M. H. & RColorBrewer, I. Package 'scales'. (2022).
24. Wickham, H., François, R., Henry, L., Müller, K. & Wickham, M. H. Package 'dplyr'. *A Gramm. Data Manip. R Packag. version* **8**, (2019).
25. Wickham, H. & Wickham, M. H. Package 'tidyr'. *Easily Tidy Data with'spread'and'gather ()'Functions* (2017).
26. Hartig, F. & Hartig, M. F. Package 'DHARMa'. *R Packag.* (2017).
27. Fox, J. *et al.* Package 'car'. *Vienna R Found. Stat. Comput.* **16**, (2012).
28. Therneau, T. M. & Lumley, T. Package 'survival'. *Surviv. Anal. Publ. CRAN* **2**, 3 (2014).
29. Bates, D. *et al.* Package 'lme4'. *URL http//lme4. r-forge. r-project. org* (2009).
30. Gentleman, R. & Winsemius, M. D. Package 'muhaz'. (2015).
31. Lenth, R., Singmann, H., Love, J., Buerkner, P. & Herve, M. Package 'emmeans'. *R Packag. version* **1**, (2019).

32. Hothorn, T. *et al.* Package 'multcomp'. *Simultaneous inference Gen. Parametr. Model. Proj. Stat. Comput. Vienna, Austria* (2016).
33. Xue, K. S., Moncla, L. H., Bedford, T. & Bloom, J. D. Within-host evolution of human influenza virus. *Trends Microbiol.* **26**, 781–793 (2018).
34. Sheen, J. K., Rasambainarivo, F., Saad-Roy, C. M., Grenfell, B. T. & Metcalf, C. J. E. Markets as drivers of selection for highly virulent poultry pathogens. *Nat. Commun.* **15**, 605 (2024).
35. Roy, B. A. & Kirchner, J. W. Evolutionary dynamics of pathogen resistance and tolerance. *Evolution (N. Y).* **54**, 51–63 (2000).
36. Fleming-Davies, A. E. *et al.* Incomplete host immunity favors the evolution of virulence in an emergent pathogen. *Science (80-.).* **359**, 1030–1033 (2018).
37. Moreno-García, M., Condé, R., Bello-Bedoy, R. & Lanz-Mendoza, H. The damage threshold hypothesis and the immune strategies of insects. *Infect. Genet. Evol.* **24**, 25–33 (2014).
38. Shaw, A. C., Joshi, S., Greenwood, H., Panda, A. & Lord, J. M. Aging of the innate immune system. *Curr. Opin. Immunol.* **22**, 507–513 (2010).
39. Izhar, R. & Ben-Ami, F. Host age modulates parasite infectivity, virulence and reproduction. *J. Anim. Ecol.* **84**, 1018–1028 (2015).
40. Ebert, D. & Weisser, W. W. Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. *Proc. R. Soc. London. Ser. B Biol. Sci.* **264**, 985–991 (1997).
41. Ben-Ami, F. The virulence–transmission relationship in an obligate killer holds under diverse epidemiological and ecological conditions, but where is the tradeoff? *Ecol. Evol.* **7**, 11157–11166 (2017).
42. Bérénos, C., Schmid-Hempel, P. & Mathias Wegner, K. Evolution of host resistance and trade-offs between virulence and transmission potential in an obligately killing parasite. *J. Evol. Biol.* **22**, 2049–2056 (2009).
43. Levin, B. R. & Bull, J. J. Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol.* **2**, 76–81 (1994).
44. Dearsly, A. L., Sinden, R. E. & Self, I. A. Sexual development in malarial parasites: gametocyte production, fertility and infectivity to the mosquito vector. *Parasitology* **100**, 359–368 (1990).
45. Leggett, H. C., Brown, S. P. & Reece, S. E. War and peace: social interactions in infections. *Philos. Trans. R. Soc. B Biol. Sci.* **369**, 20130365 (2014).
46. Messenger, S. L., Molineux, I. J. & Bull, J. J. Virulence evolution in a virus obeys a trade off. *Proc. R. Soc. London. Ser. B Biol. Sci.* **266**, 397–404 (1999).
47. Paul, R. E. L. *et al.* Experimental evaluation of the relationship between lethal or non-lethal virulence and transmission success in malaria parasite infections. *BMC Evol. Biol.* **4**, 1–12 (2004).

48. Remold, S. Understanding specialism when the jack of all trades can be the master of all. *Proc. R. Soc. B Biol. Sci.* **279**, 4861–4869 (2012).
49. Visher, E. *et al.* The evolution of host specialization in an insect pathogen. *Evolution (N. Y).* **76**, 2375–2388 (2022).
50. Wadi, L. & Reinke, A. W. Evolution of microsporidia: An extremely successful group of eukaryotic intracellular parasites. *PLoS Pathog.* **16**, e1008276 (2020).
51. Stearns, S. C. *The evolution of life histories*. **249**, (Oxford university press Oxford, 1992).
52. Bargielowski, I. & Koella, J. C. A possible mechanism for the suppression of Plasmodium berghei development in the mosquito Anopheles gambiae by the microsporidian Vavraia culicis. *PLoS One* **4**, e4676 (2009).
53. Herren, J. K. *et al.* A microsporidian impairs Plasmodium falciparum transmission in Anopheles arabiensis mosquitoes. *Nat. Commun.* **11**, 1–10 (2020).
54. Koella, J. C., Lorenz, L. & Bargielowski, I. Microsporidians as evolution-proof agents of malaria control? *Adv. Parasitol.* **68**, 315–327 (2009).

SI Appendix

Table S1. Longevity, fecundity and virulence evolution.

Table S2. Host developmental traits.

Table S3. Infection dynamics.

Table S4. Virulence decomposition.

Table S1. Longevity, fecundity and virulence evolution

Tested effect			
Model 1a - Host survival	**χ2**	***df***	***p***
Regime	138.82	2	**< 0.001**
Model 1b - Virulence	**χ2**	***df***	***p***
Regime	13.239	1	**< 0.001**
Model 1c - Cost of infection in fecundity	***df***	**F**	***p***
Regime	2	5.914	**0.003**
Day	1	20.279	**<0.001**
Replicate	4	0.695	0.596
Regime : Day	2	1.583	0.207

Table S2. Host developmental traits.

Tested effect			
Model 2a - Larvae mortality	**χ2**	***df***	***p***
Regime	7.204	2	**0.027**
Multiple comparisons for Model 2a		***z*- ratio**	***p***
Early - Late		-1.854	0.152
Early - Stock		0.729	0.746
Late - Stock		2.559	**0.028**
Model 2b - Pupae mortality	**χ2**	***df***	***p***
Regime	11.355	2	**0.003**
Multiple comparisons for Model 2b		***z*- ratio**	***p***
Early - Late		2.771	**0.015**
Early - Stock		2.520	**0.032**
Late - Stock		-0.331	0.941
Model 2c - Early pupation	**χ2**	***df***	***p***
Regime	13.763	2	**0.001**
Multiple comparisons for Model 2c		***z*- ratio**	***p***
Early - Late		3.709	**<0.001**
Early - Stock		1.744	0.189
Late - Stock		-1.987	0.116
Model 2d - Wing length	**χ2**	***df***	***p***
Regime	0.074	2	0.964

Table S3. Infection dynamics.

Tested effect			
Model 3a - Spore rate	**χ2**	***df***	***p***
Regime	4.921	2	0.085
Day	114.375	1	**< 0.001**
Regime : Day	24.854	2	**< 0.001**
Model 3b - Infection dynamics	**χ2**	***df***	***p***
Regime	41.857	2	**<0.001**
Day	158.934	1	**< 0.001**
Regime : Day	10.993	2	**0.004**

Table S4. Virulence decomposition.

Tested effect			
Model 4a - Exploitation	***df***	**F**	***p***
Regime	2	55.466	**< 0.001**
Multiple comparisons for Model 4a	***df***	***t*-ratio**	***p***
Early - Late	12	-6.086	**0.001**
Early - Stock	12	4.401	**0.002**
Late - Stock	12	10.487	**<0.001**
Model 4b - Per parasite pathogenicity	***df***	**F**	***p***
Regime	1	0.441	0.531
Day	1	2.866	0.141
Regime : Day	1	0.300	0.606
Model 4b - Virulence-transmission	***df***	**F**	***p***
ln maximum hazard	1	9.244	**0.009**