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

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Significance statement

Classical expectations on parasite evolution assume a trade-off between transmission rate and harm to the host (virulence), favoring parasites with intermediate virulence. However, recent studies challenge this idea, suggesting it is too simplistic. Here, by selecting the parasite *Vavraia culicis* for early or late transmission (or shorter and longer time within the host) in the host *Anopheles gambiae* we demonstrate that understanding virulence evolution requires considering the entirety and the type of transmission cycle and not merely the within-host stage. These findings highlight the importance of including all transmission stages in parasite evolution studies and demonstrate how certain social conditions might unintentionally select more virulent parasites.

Abstract

Evolutionary expectations about the virulence of parasites (i.e., the parasite-induced mortality rate of the host) often focus solely on the within-host transmission stage, overlooking the time spent between hosts and variations in transmission cycles. Moreover, parasite growth rate within the host is closely linked to virulence. We here suggest that a simplified view of transmission and parasite evolution makes it difficult to predict how virulence will evolve. We illustrate our ideas with a parasite with a simple life cycle, the microsporidian *Vavraia culicis*, which infects the mosquito *Anopheles gambiae*. We selected the parasite over six host generations for early or late host transmission, corresponding to shorter or longer time within the host. Selecting for late transmission increased their exploitation of the host, resulting in higher virulence and a shorter life cycle with rapid infective spore production, comparatively to selection for early transmission. In response, hosts infected with late-selected spores shortened their life cycle and shifted to earlier reproduction. Notably, higher virulence was negatively correlated with the endurance of infective spores outside the host, demonstrated in a parallel study using these parasite lines. These findings emphasize the importance of considering the entire transmission cycle in studies of parasite evolution and raise concerns about how host density and social settings might influence virulence evolution.

Introduction

Virulence is generally defined as the degree to which a parasite reduces the fitness of its host[1], commonly measured as the cost in host survival. It is the consequence of complex interactions between host and parasite strategies[2]. On one hand, the host can influence virulence 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's influence on virulence can be decomposed into processes that are linked to using its host's resources and growing (exploitation) or mechanisms that damage the host independently of the parasite's growth, such as toxins (per parasite pathogenicity)[2,5,6].

Despite the complexity of this interplay[7], most ideas about the evolution of virulence are based on simple notions: that virulence is governed by the parasite and that its evolution is constrained by its trade-off with the rate of transmission. Thus, to maximize a parasite's total transmission (that is, the rate of transmission times the period of infection), evolutionary pressure should favor 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[8,9]. While this and variations of this idea have been extensively discussed and studied for the last 50 years[8–10], empirical studies have given mixed support[11,12]. This is partly due to an overly simplistic description of transmission, the key parasite fitness measure and a vital trait for infection development and spread. As a parasite fitness trait, transmission is rather complex, since it encompasses stages within and among hosts, each with their own set of factors that can interact and trade-off against each other[13]. Nevertheless, several of these factors have been studied and indeed whether transmission is vertical or horizontal[14], continuous or not[15], or whether a parasite is directly transmitted, has environmental stages[13] , or is vector-borne[16] 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[17], 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*[18]. 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[19,20]. 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[21]. Additionally, 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.

Lastly, most studies that aspire to understand parasite evolution tend to focus on one transmission stage, either within or among hosts, and neglect the link between the two. This raises major concerns for epidemiology and the understanding of parasite and virulence evolution[13]. As expected from life history theory, a high fitness in one environment is likely to trade-off with success in other environments. This is particularly crucial for parasites, as their fitness is dependent on the success in the first host, survival in the environment they are transmitted to (e.g., water, soil, surfaces), and ability to infect a new host. Hence, it is important to relate and integrate the diverse environments a

parasite is exposed to, to fully understand its fitness and evolution and the focus on merely one of them might greatly bias our predictions regarding its evolution. For instance, a parasite that has a high fitness within one host, might not be as well equipped to survive the outside host environment, and vice-versa. Such trade-offs have been hinted at by some authors but in poorly controlled conditions. Taking this into account, we first aimed to test whether selecting for the parasite's time to transmission affects its virulence. Next, in a common garden experiment, we examined if changes in virulence due to parasite evolution influenced infection dynamics and the response of non-evolved hosts. We then integrated our results into the modern virulence decomposition framework, which allowed us to separate the parasite's impact on the host into growth-dependent (exploitation) and growth-independent (per parasite pathogenicity) costs. Finally, we connected these findings to a parallel study with the same parasite lines, which assessed differences in parasite endurance and infection ability after exposure to various temperatures and durations outside of the host.

For 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[22,23] 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, as well as within and among hosts' evolution in evolutionary and epidemiological studies. Results from this study are of universal interest for the understanding of parasite evolution and disease spread but also particularly relevant in vector-borne disease evolution where most vector-transmitted parasites require long-lived hosts or vectors to complete their life cycle.

Results

In this study we selected the microsporidian parasite *V. culicis* for early or late transmission (and shorter or longer time within the host) in the host *Anopheles gambiae* for six generations (Fig. 1a). Then, we assessed changes in parasite development within the host, as well as the host response to the parasite, when infected by the early or late-selected parasite, or upon infection with the reference stock parasite that did not undergo selection. Virulence of the infection by the different parasites was also measured and decomposed into exploitation (cost dependent due to parasite growth) and per parasite pathogenicity (cost independent of parasite growth). It is noteworthy to emphasize virulence in this study is measured as a cost in host survival.

Concerning the parasite, we quantified its: i) cost in host survival, or virulence (Fig. 1bc); ii) cost in host fecundity (Fig. 1d); iii) spore rate and dynamics (Fig. 3); and iv) decomposed the parasite contribution for virulence through the quantification of cost dependent and independent of growth in host survival (Fig. 4). Regarding the host, we measured: i) its fecundity shift in response to the different parasites (Fig. 1d); and ii) developmental time and mortality during the larval and pupal

stage upon infection (Fig. 2). Altogether, with this set of results, we aimed to understand the drivers of parasite and virulence evolution within the host stage and how they contribute to host infectivity.

Selection for late transmission increases virulence and shifts host fecundity

The selection regime had a cost in host longevity, with the individuals infected with spores selected for late transmission living less than individuals infected with early-selected or unselected spores ($\chi2 = 138.82$, $df = 2$, $p < 0.001$; Fig. 1b). The comparison of the maximum hazard (a proxy for virulence) 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). Both of these results suggest that selection for later transmission, and consequently longer time within the host, selects for more virulent parasites which greatly reduce the lifespan of the host, in comparison with infection by early-selected or unselected spores (Fig. 1bc). See SI Appendix Table S1 for further details on these analyses.

When accounting for the cost of infection in fecundity, we observed this was strongly affected by the selection regime as well ($df = 2$, F= 5.914, $p = 0.003$; Fig. 1d). However, in this case, infection by both early- and late-selected parasites led to a lower cost for the host in comparison to infection by the unselected reference parasite. Nevertheless, the cost of infection in host fecundity was also affected by day ($df = 1$, F= 20.280, $p < 0.001$; Fig. 1d), with the cost increasing from day 10 to 15 of host adulthood. Although there was no significant effect of an interaction between the selection regime and assay day, a multiple comparisons test was still performed to measure differences between each treatment for each day. Hence, we observed that the cost in fecundity in individuals infected with late-selected parasites on day 10 was less severe than either that of individuals with late parasites on day 15 of adulthood ($df = 258$, t-ratio = 4.164, $p<0.001$) or unselected parasites on day 10 ($df = 258$, t-ratio = 3.533, $p<0.001$) and 15 ($df = 258$, t-ratio = 4.916, $p=0.006$). In sum, these results suggest infection by selected parasites had a lower cost in fecundity in comparison to infection by the unselected one. Also, the cost in fecundity increased with infection progression, in particular for individuals infected with late-selected parasites, where we observed an increase in fecundity for the first day, suggesting a shift in fecundity by the host. Further statistical details on SI Appendix Table S1.

Selection shortened host and parasite life cycles

Afterward, we focused on parasite-driven changes in host development (Fig. 2). First, we compared larvae mortality between selected and unselected parasites, and then between early and late-selected parasites. We did not observe any statistical difference for either of the comparisons, showing parasite selection does not affect larvae mortality (Fig. 2a). However, at the pupal stage, we observed that a host infected by an early-selected parasite was more likely to die in this stage, comparatively to hosts infected by late-selected parasites ($\chi2 = 7.890$, $df = 1$, $p = 0.005$; Fig. 2b). Nevertheless, selection for

late transmission favored parasites that shortened the host time to pupation, when compared to early transmitted ones ($\chi2 = 14.267$, $df = 1$, $p < 0.001$; Fig. 2c). Although the last result provides evidence of a parasite-driven shortening of host developmental time, such outcome had no consequence in adult host body size ($\chi2 = 0.054$, $df = 1$, $p = 0.816$; Fig. 2d). We believe the shortening of the host cycle is considerably small and therefore, does not substantially affect the host growth. Further details can be found in the SI Appendix Table S2, and replicate variation is presented in Figure S1.

We then turned our focus to changes in infection parameters, such as spore rate (proxy for when the infective stages are produced) and their respective number produced per day (severity of infection and individual infectiousness). First, the spore rate increased with time ($\chi2 = 114.375$, $df = 1$, $p < 0.001$) as it is expected. However, this increase was stronger for parasites selected for late transmission and weaker for unselected parasites (interaction between selection regime and day: $\chi2 = 24.854$, $df = 2$, $p < 0.001$) (Fig. 3a), meaning late-selected spores shortened their life cycle and started producing infective spores much sooner than the other treatments. The number of spores produced per day was also affected by the 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 produced increased with the progression of infection and did so more strongly if the parasites had been selected for late transmission (Fig. 3b). Additional details are in SI Appendix Table S3.

An increase in host exploitation underpins virulence evolution

We then aimed to relate the latter changes in infection dynamics to the parasite's respective virulence. For this we decomposed virulence into two components: a) exploitation, i.e. cost in host survival due to parasite growth; b) per parasite pathogenicity, i.e., cost in host survival independent of parasite growth. This decomposition allows us to understand if parasite evolution is due to changes in the parasite growth rate, or pathogenicity, such as the acquisition of virulence factors or toxin production. In our case, 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. 4ab; See SI Appendix Table S4 for statistical comparisons). These findings suggest 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 slows down around days 15 and 18 of adulthood, almost resembling a plateau, we used spore density as a proxy of individual infectiousness and tested for a relationship between infectiousness and virulence in this model system. It is important to mention that this sort of rationale is valid for the parasite model in question, as our measure of parasite growth is the number of infective cells that will be transmitted to the environment, and consequently infect future hosts. Therefore, for this model, spore growth is equivalent to host infectiousness, one of the transmission stages discussed in Silva *et al.* (2024). Using a linear model, we detected an effect of virulence (i.e., natural log of the maximum hazard) on host infectivity (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 host infectivity, or spore growth, and virulence in this system.

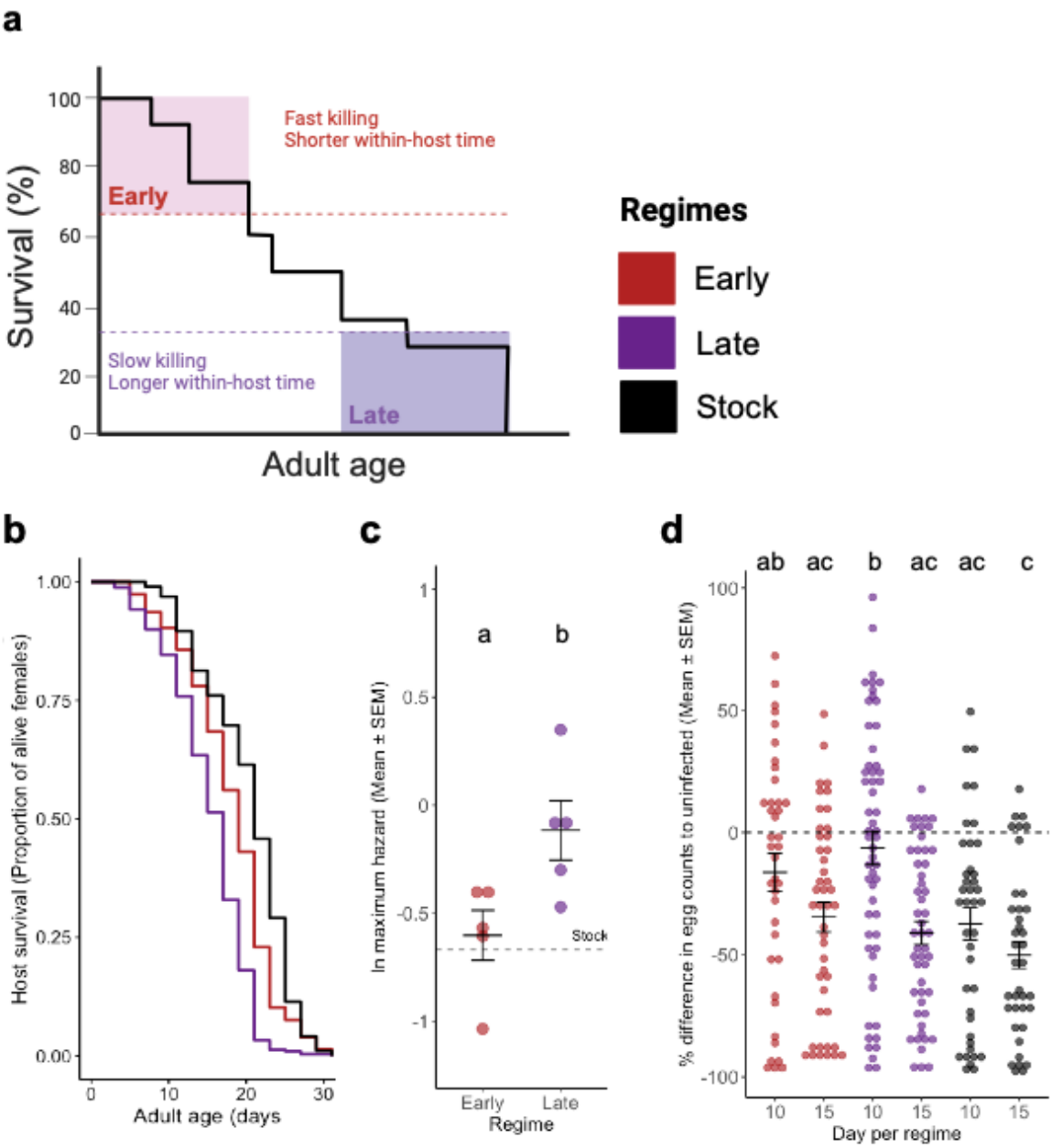

Figure 1. Longevity, fecundity and virulence evolution. (a) Experimental design, describing direct and indirect forms of parasite selection in this study. **(b)** Host survival after infection with evolved or reference parasites. Early and Late curves were generated with 487 and 519 females split throughout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

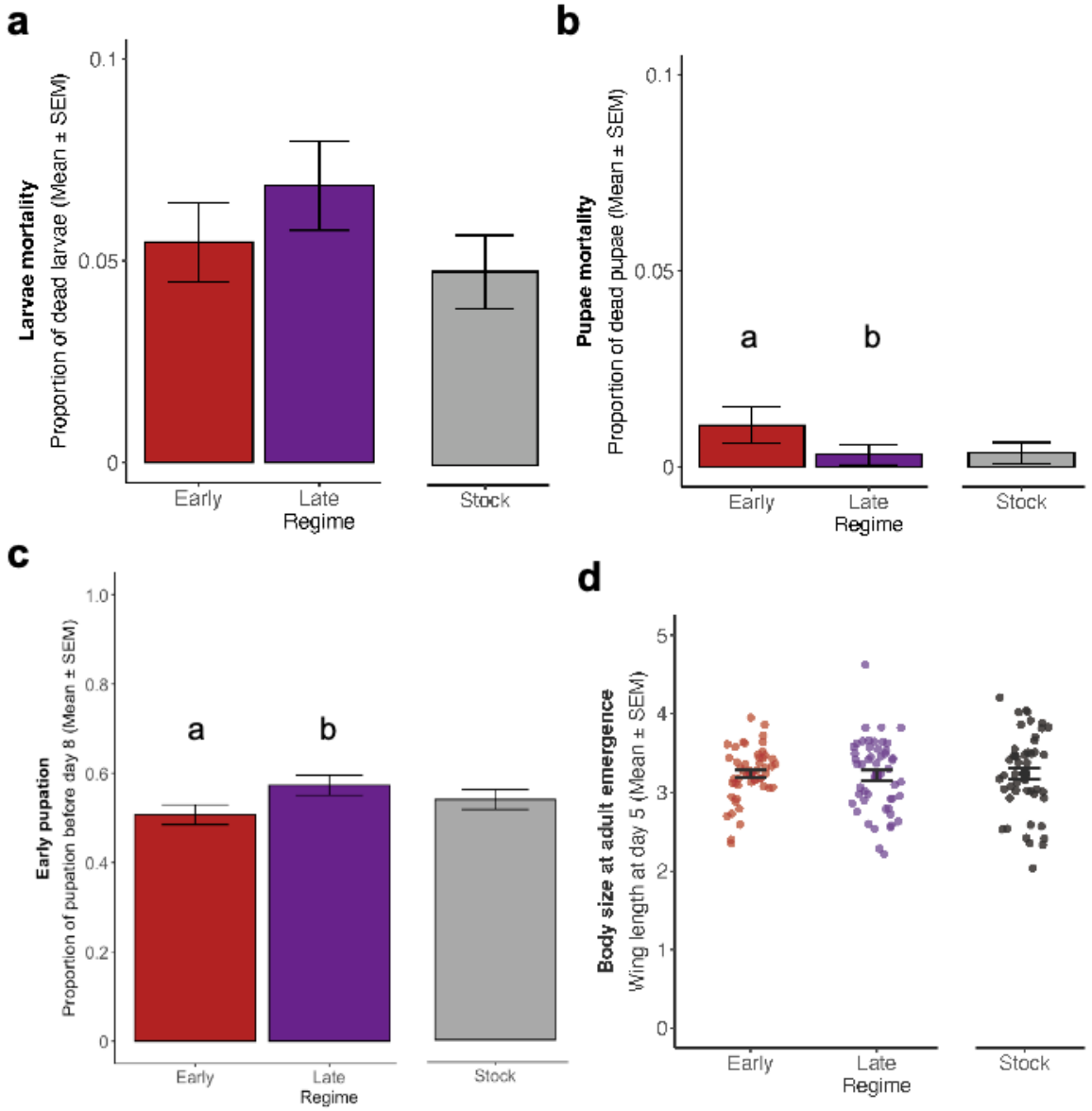

Figure 2. Host developmental traits. Selected regimes (i.e., early and late) were analyzed together with and separately from the stock. **(a)** Larvae mortality across regimes. The regimes were composed of 2068, 2011 and 2037 individuals for Early, Late and Stock. **(b)** Pupae mortality with each regime 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 and Figure S1 for mean and standard error for each of the replicates.

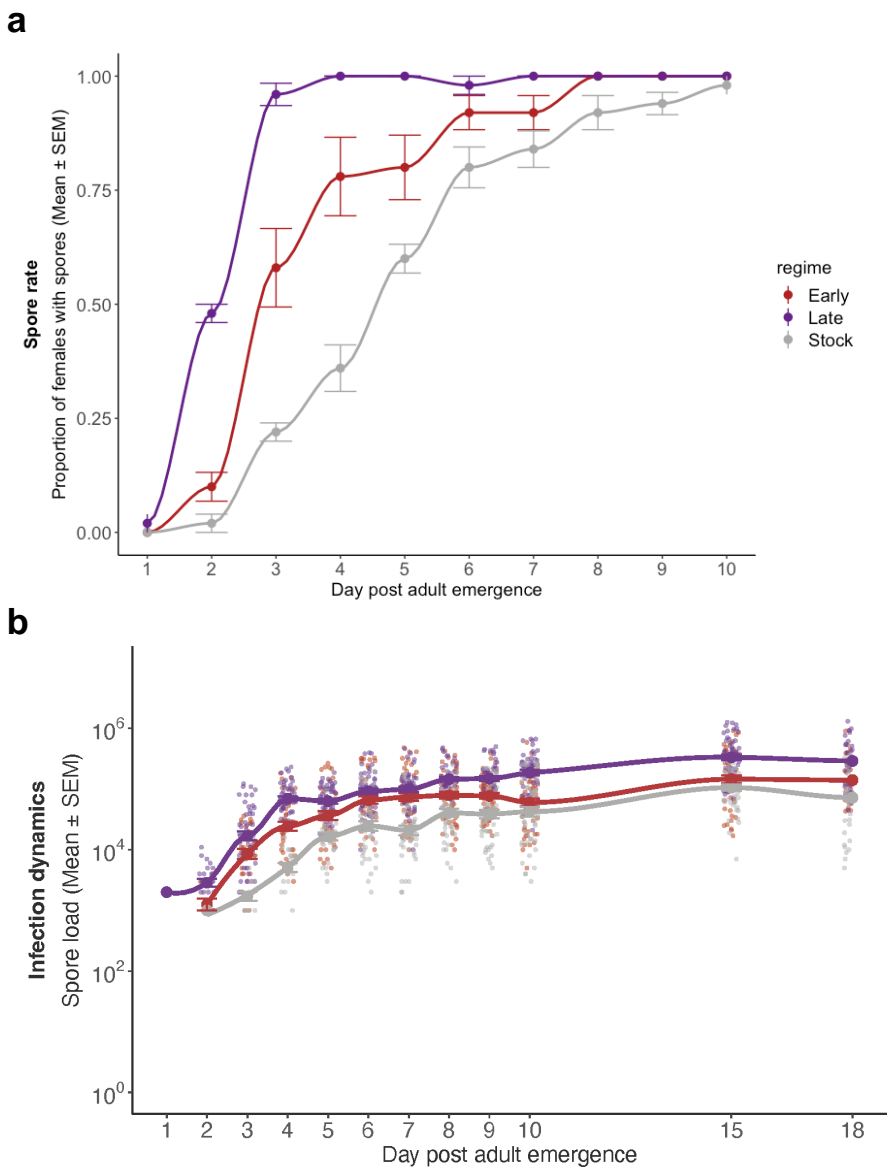

Figure 3. Infection dynamics. (a) Spore rate, as the daily proportion of females with detectable spores for each treatment during the first ten days of adulthood. **(b)** Spore dynamics, as the mean number of spores per female for each of the first ten days, as well as days 15 and 18 (i.e., chronic load).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

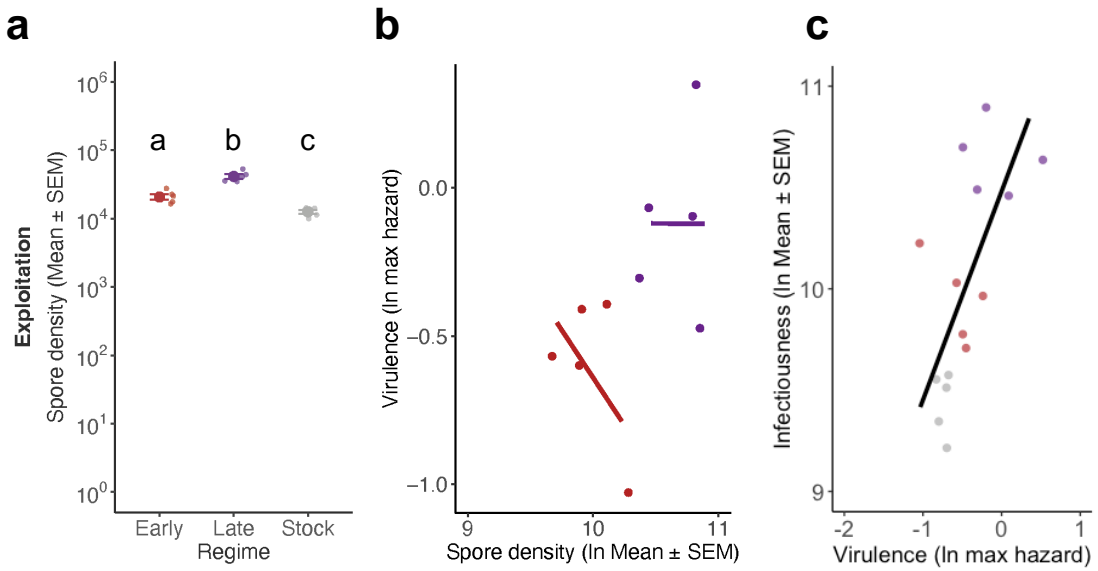

Figure 4. Virulence decomposition. (a) Exploitation was measured as the mean spore density at days 15 and 18 (i.e., spore load divided by wing size) for every replicate of each regime. **(b)** Per parasite pathogenicity, as the relationship between virulence and spore density. **(c)** Infectiousness-virulence relationship for this infection model. Given that in this infection model, the spore load is expected to decelerate 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 can transmit. There is a positive relationship between virulence and host infectiousness ($df = 1$, $F = 31.534$, $p < 0.001$). Different letters denote means that they are significantly different from one another. See SI Appendix Table S4 for further details on the statistical analysis.

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[17], a longer time within the host (i.e., late transmission) was selected for greater virulence than a shorter time (i.e., early transmission). Furthermore, we provided contrasting evidence to a study on nuclear polyhedrosis virus (NPV) infections in the moth *Lymantria dispar*[18], in which selection of the virus for late transmission led to lower virulence (and higher viral burden) than selection for early transmission. Below we argue and explain why these two contrasting findings might reflect current limitations in the study of parasite evolution. We also demonstrate that our study serves as a suitable example to reflect on classical theories of parasite evolution and how the integration of parasite life history is vital for the understanding of its evolution and consequently, virulence itself.

The different outcomes observed in these two studies emphasize the complexity of the evolution of virulence[7,12,13]. In particular, it may result from the different levels at which selection operates on parasites: i) competition between parasites within one host; ii) the ability to survive an environment

outside the host; iii) or transmission among hosts[13]. The interlinking of these, and dependent traits, can either limit or facilitate their evolvability, which in turn has major consequences for the understanding of virulence evolution. Indeed, if we only consider selection at the level of transmission among hosts, as assumed by much of the theory of the evolution of virulence[8,9] and by Osnas and Dobson (2010), selection for early transmission would lead to a higher killing rate than selection for late transmission.

However, if we instead consider sources of selection within the host, we can expect a different evolutionary outcome, which in turn aligns with our results.

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 (an unlikely explanation for our results) or the strength of density-dependence as the parasite load increases, and thus increases the possibility of competition among parasite genotypes[24,25], in a phenomenon described as clonal interference[19]. This selects for more transmissible or more rapidly replicating parasites in our study (Fig. 3 and 4) and others[19,24]. 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[26]. We expect this phenomenon to happen not only in poultry markets with lower turnover but also in society and the wild, and literature has reflected this trend[25]. 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[27], thus hindering the fixation of genotypes with low virulence[28]. In addition, the host's immune response should increase at higher parasite burdens[6,29] 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[30,31], 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 good example of this rationale is provided by the evolutionary models about obligate killers[12,32–34] , which assume that the host is a limited resource, making the parasite's growth density-dependent and decoupled from its transmission. This makes it possible for "selfish" genotypes to grow without counter-selection[32,35]. In our study, although *V. culicis* is not an obligate killer (see Materials and Methods), the experimental design made it behave like one, by restricting its transmission route, and therefore, both assumptions are likely to hold in our experiment. In particular, our protocol eliminated many costs of transmission. Thus, for example, since spores (infective stage of our parasite) 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[12,36,37]. Indeed, the positive correlation between chronic spore load (i.e., a proxy for infectiousness) 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[5,38,39].

This hypothesis introduce us to the third level at which selection can act on: the ability to endure the environment among hosts, a transmission stage common to most parasites[13,38,40]. This resting stage is crucial for *V. culicis* because its transmission cycle encompasses death of an individual on water and release of infective spores that will be ingested by young larvae. These spores can sit and wait for a few hours to a several months[41], making their survival in the external environment essential for a successful transmission. In respect to this, a follow-up study of ours with these same evolved lines quantified the parasites' ability to endure external conditions and their resilience to different temperatures over time [42]. Consistent with life history theory, we found that spore growth within the host was negatively correlated with survival in the external environment. This meant that spores selected for late transmission, which had the highest growth rate within a host, were the most susceptible to dying in the external environment. This effect was more pronounced with longer exposure to the outside environment and at higher temperatures. In contrast, early-selected and unselected reference spores remained highly viable for the whole duration of the experiment, i.e., 90 days. A reduction in parasite viability in late transmitted spores led to lower infectivity in new hosts and less severe infections with lower parasite burden compared to their counterparts. Hence, we demonstrated that selection within the host can inadvertently impact parasite fitness outside of the host. This is of particular significance from a social perspective, as increased host density due to scenarios with social crowding or aggregation behaviors might shorten the time parasites spend in the external environment. This favors parasites that prioritize evolution within the host stage, and which invest in host exploitation and within-host growth.

Several other results in this study 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 specialize on a single host without being impeded by the cost of having to also perform on a second one[43–45]. However, selection did not affect per-parasite pathogenicity for any of the regimes (i.e., damage independent of parasite growth such as production of toxins, Fig. 4b). This is likely due to the limited microsporidian genome[46,47] and, to our knowledge, lack of virulence factors on which selection can act.

Second, our selection protocol had an impact on some aspects of the mosquitoes' development. For instance, infection by late-selected parasites led to a shortening of the host life cycle through early pupation (Fig. 2c). This result is likely due to the properties of this parasite treatment. Indeed it has

been hypothesized infection by more virulent parasites induces strong stress responses that activate higher levels of immunity and shorten the host developmental time[6,48]. This hypothesis is supported by our results, as the most virulent parasite (late-selected) shortened the host developmental time (Fig. 2c), while the less virulent parasite (early-selected) did not, and instead carried a cost in pupae mortality (Fig. 2b). Nevertheless, neither of these changes was big enough to impact body size at adult emergence (Fig. 4d).

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[49], 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 whole parasite life cycle to understand its evolution. The findings and arguments presented here have the intention to make the reader reflect on classical theories and its applicability into modern society and disease spread strategies. We validate many of the concerns previously discussed in literature concerning the current theory of virulence evolution and how they link to the oversimplification of parasite transmission, the main parasite fitness measure. Although usefully simplistic, the current theory of virulence disregards a considerable portion of parasite strategies, which encompass different transmission stages that evidently cannot be neglected, even when solely studying within host aspects of infection.

Importantly, we believe this study has major consequences for epidemiology, vector-control and disease spread strategies, regardless of the host-parasite model system in question. The latter is due to the nature of parasites' transmission cycles, which as discussed in detail by Silva *et al.* (2024) present the same transmission stages regardless of the taxa, and therefore evolutionary outcomes and interactions discussed here can be adapted and further explored in most parasite taxa. Moreover, one of the key take-home messages is the importance of the balance between within-host and among host evolution. This has obvious consequences for disease evolution and spread, as it has become increasingly obvious and alarming in recent pandemics, such as the SARS-CoV2, with the rise of disproportionately high transmission associated to higher virulence in certain social settings[50–52]. Only through the understanding of this balance, can we predict and design better disease control strategies. Undeniably, a continued short-sighted view of parasite evolution with within-host evolution as the sole focus of the field will hinder our understanding of the parasite evolution and promulgate the controversy between virulence and transmission.

Lastly, we believe is imperative to make a small nod to the importance of these findings to the ecology of this parasite. Microsporidia, such as the one in this study, have been proposed as biological agents for malaria control[22,53] due to their ability to suppress *Plasmodium* infections in

377 wild- and lab-grown mosquitoes[54,55]. In line with this, is possible that the geographical distribution of
378 mosquitoes (and microsporidia they carry) might influence microsporidia evolution and therefore its
379 control potential. Surely, infection by our microsporidia is unlikely to affect mosquito population
380 numbers, as our late-selected treatment with shorter lifespan has great earlier fecundity (Fig. 1d), but
381 the same cannot be expected regarding wild mosquito density. Although, the mechanisms
382 underpinning *Plasmodium* suppression are still unknown, it is fair to expect microsporidia evolution
383 to trade-off with its ability to interfere with *Plasmodium* development to a certain extent. Hence, this
384 system seems a unique model to test microbe-driven vector-control mechanisms, while allowing to
385 study of fundamental evolutionary questions regarding infection and microbe interactions.
386

387 # Materials and methods

388 *Experimental model*

389 We used the microsporidian parasite *Vavraia culicis floridensis*, provided by James Becnel (USDA,
390 Gainesville, FL) and the Kisumu strain of the mosquito host *Anopheles gambiae (s.s)*. Mosquitoes
391 were reared under standard laboratory conditions (26 ± 1ºC, 70 ± 5% relative humidity and 12 h
392 light/dark). *V. culicis* is a generalist, obligatory, intracellular parasite that infects mosquito epithelial
393 gut cells. Mosquitoes are exposed to the microsporidian during the larval aquatic stage, where they
394 ingest spores in the water and food. After replication within the larvae, they start producing spores
395 from within the adult gut cells, re-infecting new cells and propagating the cycle until they kill the
396 host. Spores are transmitted when infected individuals die in water or from mother to offspring by
397 adhering to the surface of the eggs. Before starting the experiment, *V. culicis* was kept in large
398 numbers by alternatingly infecting Aedes *aegypti* and *An. gambiae* populations, to ensure it remains a
399 generalist parasite. The parasite was allowed to develop within these populations until host death
400 when *V. culicis* spores were retrieved for later infections.
401

402 *Experimental evolution and rearing*

403 A starting population of 300 freshly hatched *An. gambiae* larvae were grown in groups of 50 per petri
404 dish (120 mm diameter x 17 mm) containing 80 mL of distilled water. Larvae were fed with daily
405 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,
406 2, 3, 4 and 5 days or older, respectively. On the second day of development, individuals were exposed
407 to 10,000 spores of *V. culicis* per larva. Upon pupation, individuals were transferred to cages (21 x 21
408 x 21 cm) and left to emerge into adults. Freshly prepared cotton pads with 6% sucrose solution were
409 provided throughout their adult life. At adulthood, two selection regimes were generated (Fig. 1a): i)
410 Early transmission, where the spores from the first 50 females to die were retrieved, mixed and used
411 to infect the following generation of the same regime; ii) and late transmission, where the spores from
412 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 pooled all selected individuals, 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 concentration of the resulting spore solution 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.

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 [14]. 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.

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”[56] , "purrr"[57], "scales"[58], "dplyr"[59] and "tidyr"[60], as well as created with BioRender.com and edited in Microsoft PowerPoint 16.16.27. Statistical analysis and their diagnosis were conducted using: “DHARMa”[61], “car”[62], “survival”[63], "lme4"[64], "muhaz"[65], "emmeans"[66] and "multcomp"[67] .

449 Longevity was compared across regimes using a cox proportional hazard with the regime as the
450 explanatory variable and the replicate as a random factor. Then, we generated a survival curve for
451 each replicate of each regime and extracted its maximum hazard, as in[6], our proxy for virulence.
452 Differences in virulence were assessed through a linear model with the natural log of the maximum
453 hazard as a dependent variable, the regime as the explanatory variable and the replicate as a random
454 factor.
455 Concerning the cost of infection in fecundity, we first tested if the proportion of non-egglaying
456 females was the same across all regimes and days for each replicate. For this, we ran a generalized
457 linear model with a binomial structure and both regime and day post-adult emergence as fixed factors,
458 as well as their interaction, and replicate as a random factor. There was no effect of any of the
459 treatments and therefore, only considering females who had laid eggs, we calculated the percentual
460 cost of infection by subtracting the mean uninfected fecundity from each infected individual
461 fecundity, for day 10 and 15 of adulthood, and dividing the resulting value by the infected individual
462 fecundity and multiplying it by 100. Then, we assessed differences due to regime and/or days post-
463 adult emergence using a linear model with replicate as a random factor.
464 Larvae and pupae mortality were measured as the proportion of dead individuals, for each of the
465 stages. A generalized linear model with a binomial error structure, and the replicate as a random
466 factor, was used to determine the effect of regime on each of the developmental stage mortality.
467 Individuals pupated from day 6 to 11 post eclosion, but approximately 77%, 79% and 82% of them
468 pupated on day eight or nine, for Early, Late and Stock, respectively. Therefore, we split individuals
469 into two categories, the ones that pupated until day eight and the ones that pupated from day nine
470 onwards and used a generalized linear model with binomial distribution to measure differences in
471 early pupation. As before, the regime was considered an explanatory variable and the replicate as a
472 random factor.
473 We defined spore rate as the proportion of females with detectable spores per regime for each of the
474 first ten days of adulthood. Our detection threshold is estimated to be 100 spores. Mosquitoes with
475 detectable spores were then used to estimate spore dynamics from day one to 18 of adulthood. The
476 spore rate was analysed using a generalized linear model with a binomial distribution, while for spore
477 dynamics we used a linear model to assess differences in the log-transformed number of spores. In
478 both models, we had regime and day, and their interaction, as explanatory factors and the replicate as
479 a random factor.
480 To decompose virulence, we used the maximum hazard from the longevity curves and (since the
481 average maximum hazard day was 20) the spore density on days 15 and 18. Spore density was
482 calculated as the individual spore load at days 15 and 18 adjusted by individual wing length.
483 Virulence decomposition analysis was done as in [6]. In summary, exploitation was measured as the
484 natural log-transformed mean spore density from days 15 and 18 for a given regime and replicate,
485 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.

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.

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Competing Interests

The authors declare that they have no competing interests.

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Supplementary Information

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

by Luis M. Silva and Jacob C. Koella

Table S1. Longevity, fecundity and virulence evolution.

Table S2. Host developmental traits.

Table S3. Infection dynamics.

Table S4. Virulence decomposition.

Figure S1. Host developmental traits per replicate.

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	χ2	*df*	*p*
Model 2a - Larvae mortality			
Selected *vs*. Stock:			
Selection	3.564	1	0.059
Early *vs.* Late:			
Regime	3.437	1	0.064
Model 2b- Pupae mortality			
Selected *vs*. Stock:			
Selection	2.324	1	0.127
Early *vs.* Late:			
Regime	7.890	1	**0.005**
Model 2c - Early pupation			
Selected *vs*. Stock:			
Selection	0.010	1	0.920
Early *vs.* Late:			
Regime	14.267	1	**< 0.001**
Model 2d - Body size			
Selected *vs*. Stock:			
Selection	0.027	1	0.869
Early *vs.* Late:			
Regime	0.054	1	0.816

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	0.537
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**

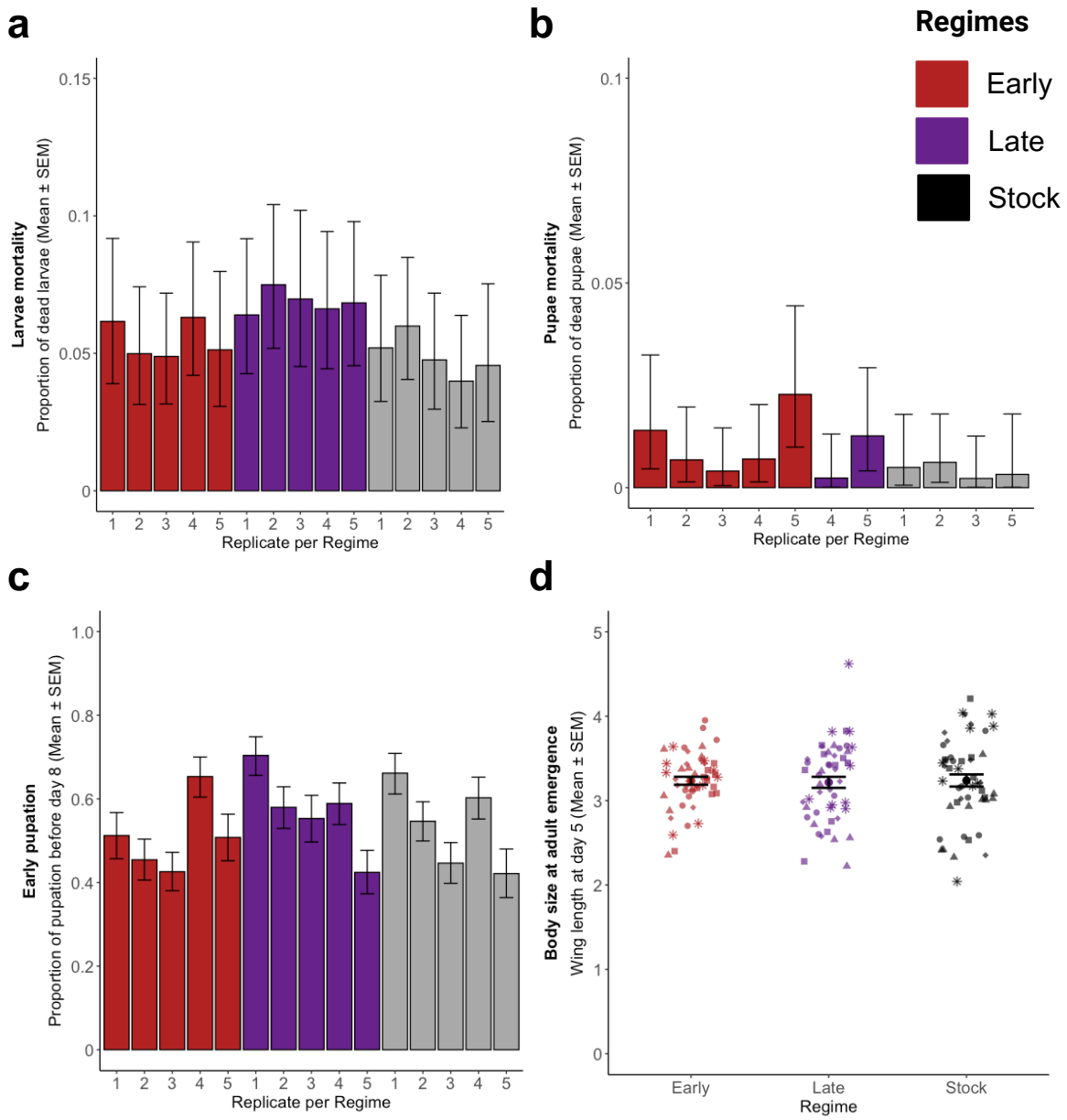
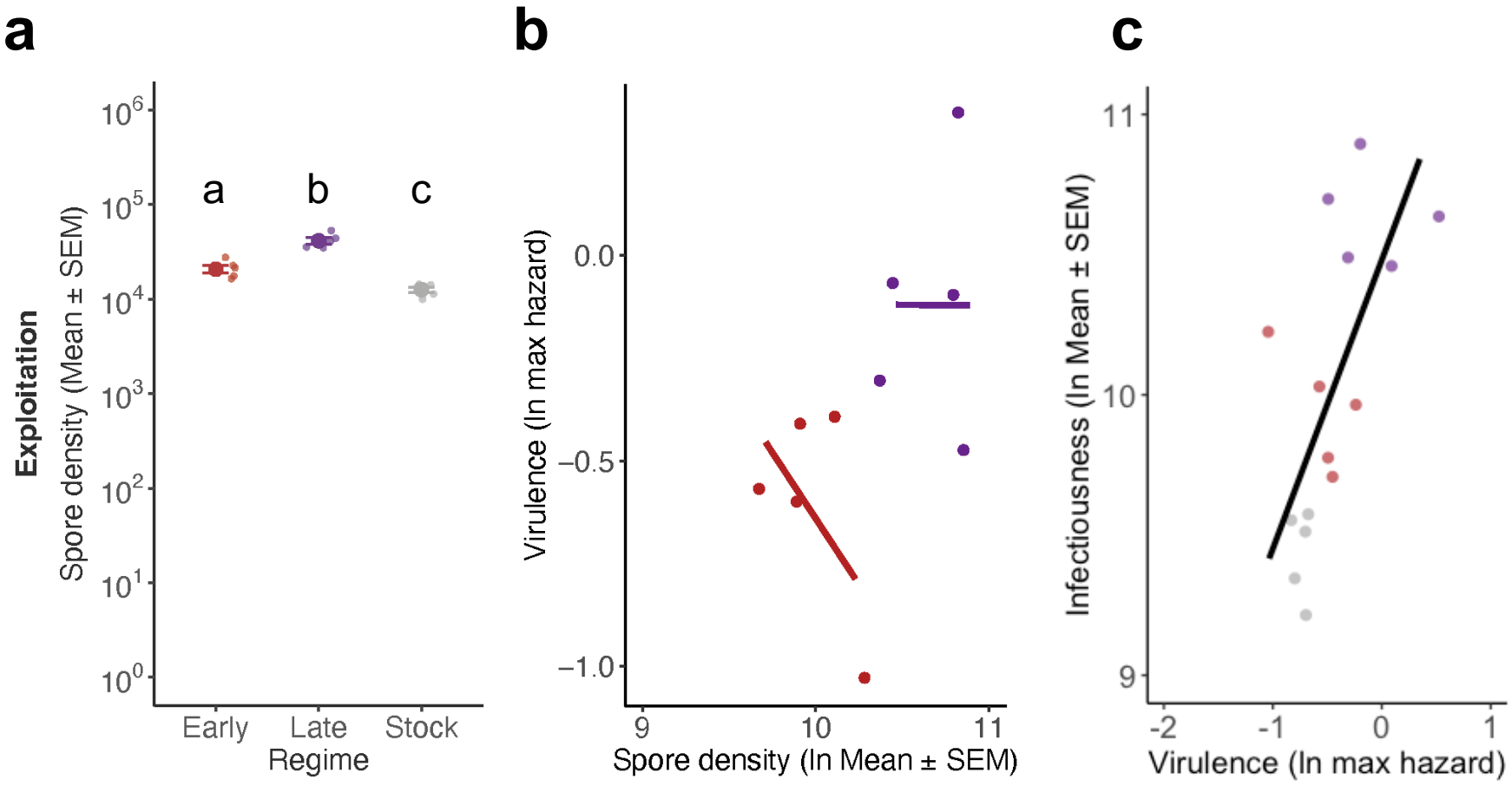
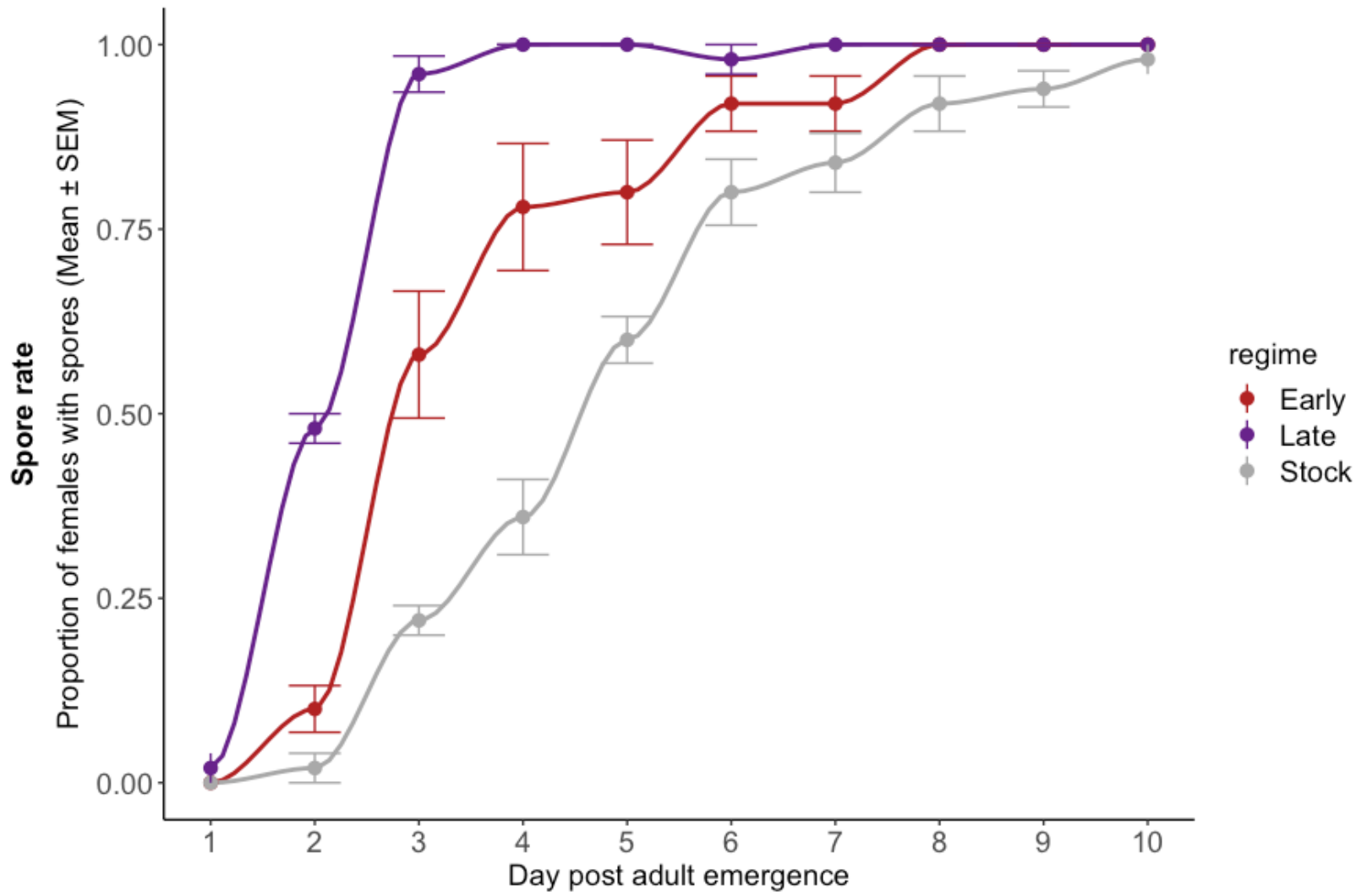


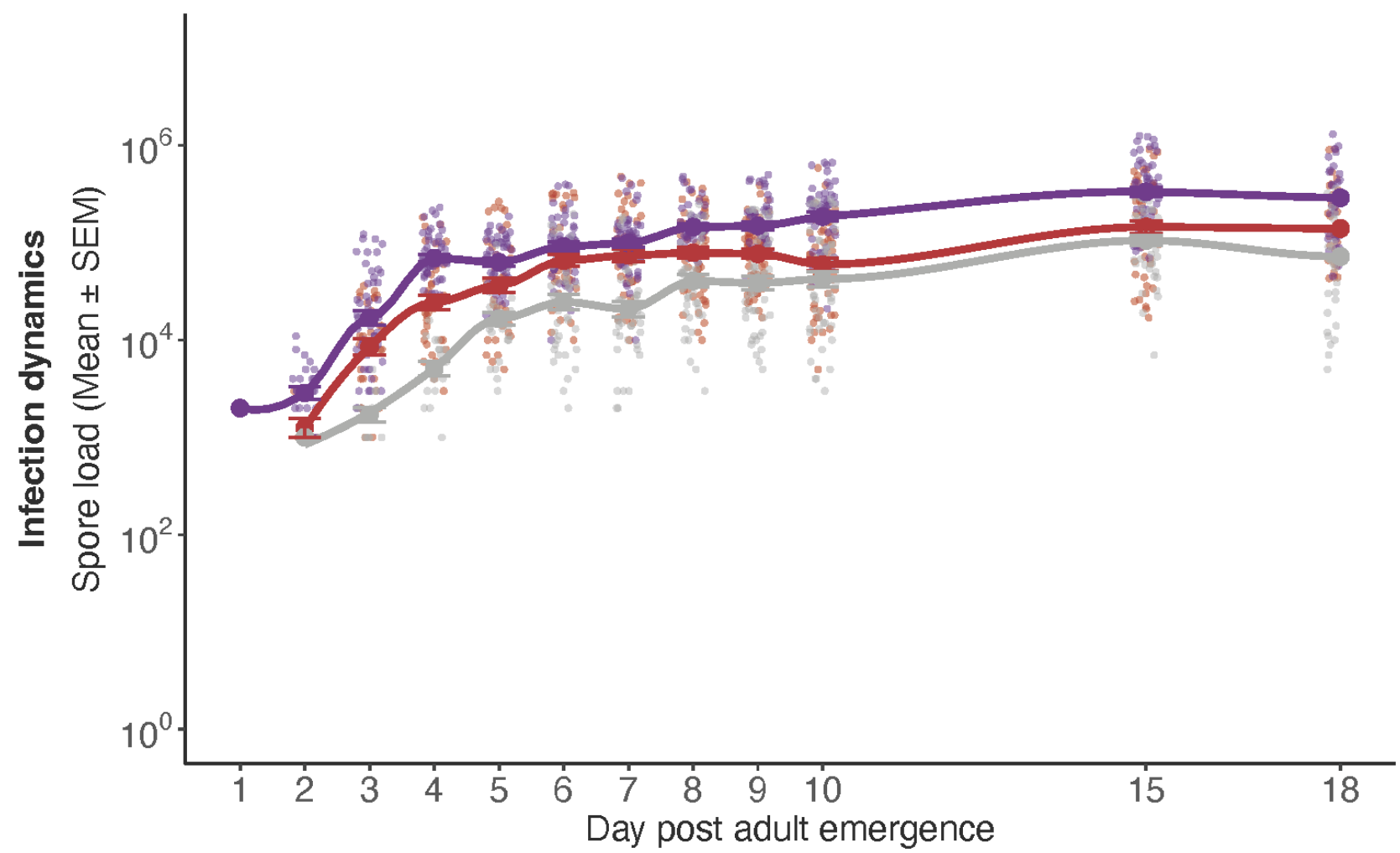
Figure S1. Host developmental traits per replicate. (a) Larvae mortality across regimes and replicates. Sample size as follows: Early with 357, 441, 491, 428 and 351: Late with 422, 427, 344, 423 and 395; Stock with 404, 484, 441, 401 and 307 for replicate 1 to 5 respectively. **(b)** Pupae mortality for each regime and replicate. Missing bars represent replicates with no pupae mortality. Sample size was: Early with 335, 419, 467, 401 and 333: Late with 395, 395, 320, 395 and 368; Stock with 383, 455, 420, 385 and 293 for replicate 1 to 5 respectively. **(c)** Early pupation, as the proportion of individuals that pupated until day eight post eclosion. Sample size was the following: Early with 330, 416, 465, 398 and 325: Late with 395, 395, 320, 394 and 363; Stock with 381, 452, 419, 385 and 292 for replicate 1 to 5 respectively. **(d)** Body size at adult emergence using wing length of five-day-old females as a proxy. Each replicate consisted of 10 individuals, illustrated by a different dot shape.

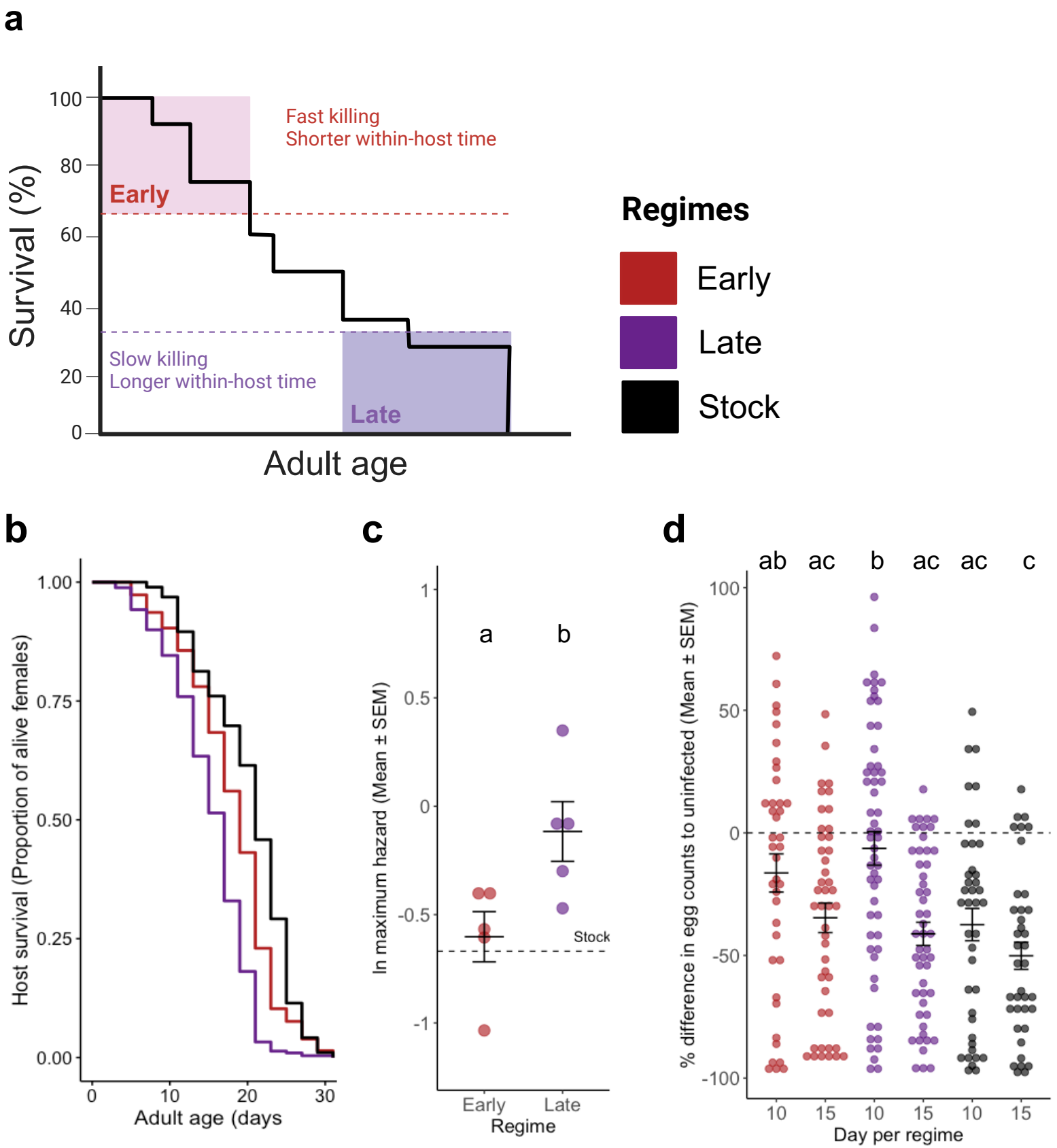

a
b
c
Exploitation
Spore density (Mean ± SEM)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
a
b
c
Early
Late
Stock
Regime
Virulence (ln max hazard)
0.0
−0.5
−1.0
9
10
11
Spore density (ln Mean ± SEM)
Infectiousness (ln Mean ± SEM)
11
10
9
-2
-1
0
1
Virulence (ln max hazard)

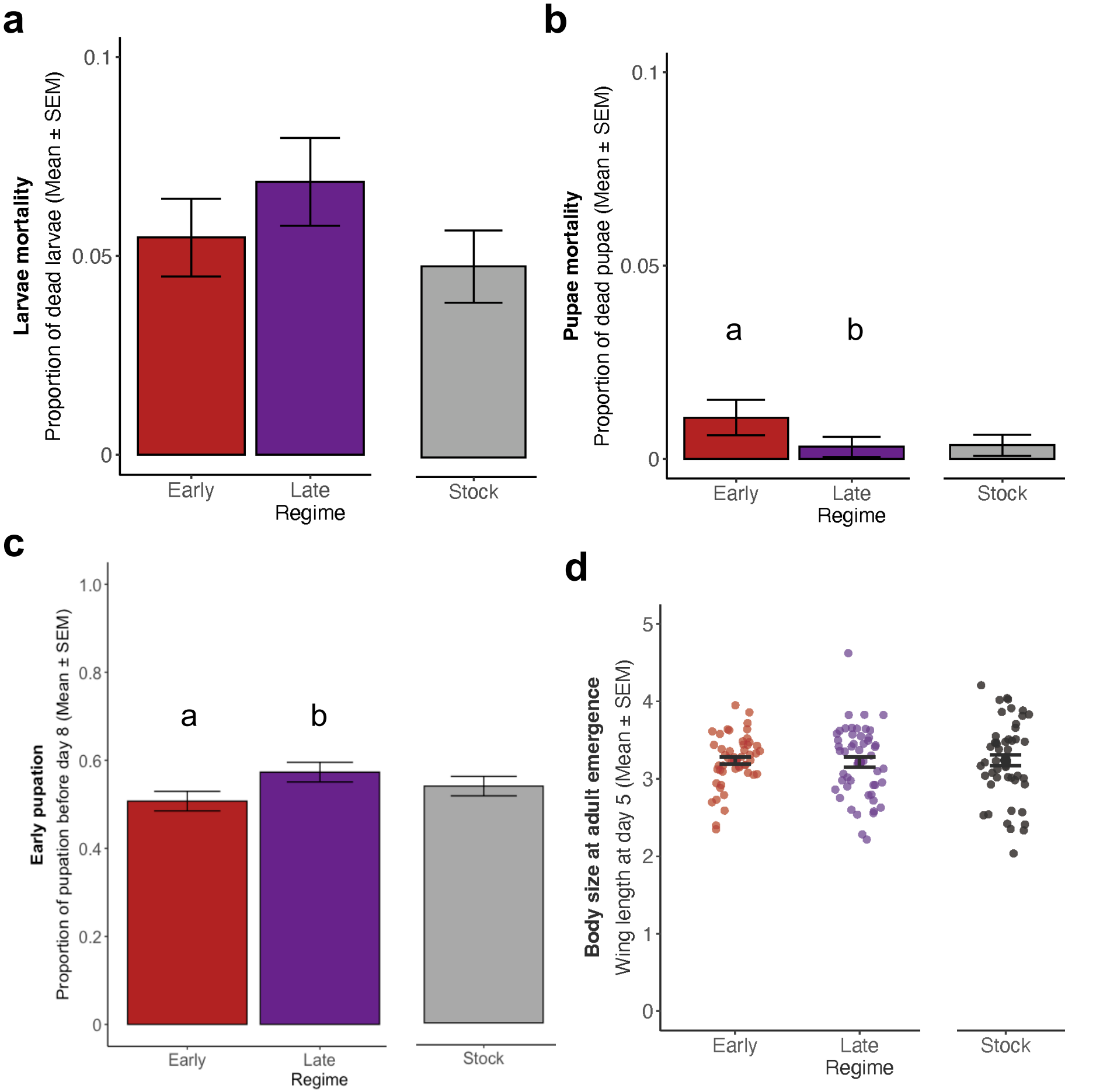
a


Spore rate
Proportion of females with spores (Mean ± SEM)
1.00
0.75
0.50
0.25
0.00
1
2
3
4
5
6
7
8
9
10
Day post adult emergence
regime
Early
Late
Stock

b


Infection dynamics
Spore load (Mean ± SEM)
10^6
10^4
10^2
10^0
1
2
3
4
5
6
7
8
9
10
15
18
Day post adult emergence


a
Survival (%)
100
80
60
40
20
0
Fast killing
Shorter within-host time
Early
Slow killing
Longer within-host time
Late
Adult age
Regimes
Early
Late
Stock
b
Host survival (Proportion of alive females)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Adult age (days
c
ln maximum hazard (Mean ± SEM)
1
0.5
0
-0.5
-1
a
b
Stock
Early
Late
Regime
d
ab
ac
b
ac
ac
c
% difference in egg counts to uninfected (Mean ± SEM)
100
50
0
-50
-100
10
15
10
15
10
15
Day per regime


a
Larvae mortality
Proportion of dead larvae (Mean ± SEM)
0.1
0.05
0
Early
Late
Stock
Regime
b
Pupae mortality
Proportion of dead pupae (Mean ± SEM)
0.1
0.05
0
a
b
Early
Late
Stock
Regime
c
Early pupation
Proportion of pupation before day 8 (Mean ± SEM)
1.0
0.8
0.6
0.4
0.2
0
a
b
Early
Late
Stock
Regime
d
Body size at adult emergence
Wing length at day 5 (Mean ± SEM)
5
4
3
2
1
0
Early
Late
Stock
Regime