Virulence evolution: thinking outside of the host

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Abstract

The main theory of the evolution of virulence relies on a trade-off between virulence and the rate of transmission. It has however proven difficult to measure the required trade-off. This lack of support may partly be due to the lack of information about the parasite's behaviour in the environment between hosts, where the parasite finds itself during transmission. In this study, we used the parasite *Vavraia culicis* and its host, the mosquito *Anopheles gambiae* to investigate the interplay between the parasite's virulence in its host and its longevity outside its host. To do so, we selected parasite lines for early or late transmission and used a transmission decomposition framework to describe several traits of the parasite's life cycle at two temperatures. Lines with greater virulence and that grew more rapidly within their host lived less long in the external environment, irrespective of temperature. These results underscore the importance of considering the full context of transmission and other parasite fitness traits in studying and predicting the evolution and spread of infectious diseases.

Introduction

The evolution of virulence (i.e., parasite-induced decrease of the host's fitness) is a central topic in evolutionary biology due to its ramifications for fields such as epidemiology and medicine. This is because it lays the foundations for many aspects of conservation biology and the epidemiology of infectious diseases [1]. The prevailing theory of virulence evolution relies on a trade-off between the parasite's virulence and rate of transmission [2, 3], and therefore often predicts that virulence is optimal at intermediate levels. This simple theory is intuitive and helpful and has therefore found support from the research community [4–7], although its limited evidence about its trade-offs and predictions from studies in experimental or natural populations has also led to scepticism about the idea [1, 8–13]. This scepticism can be explained by the following few points. The first is one of the consequences of what the theory predicts, i.e., that the parasite's virulence is optimal at intermediate levels, as a higher virulence (i.e., higher host mortality) reduces chances of transmission by cutting short the parasite's transmission and life cycle. This is hard to test, as in wild conditions it will be very unlikely to observe parasites with high virulence and low transmission, as naturally, this scenario does not reflect the parasite's interests and instead would reduce its fitness. The second point is the diversity in the parasite's life cycles and transmission strategies. It is difficult to compare parasites, and in particular compare the concepts of their trade-offs, if they have horizontal *vs.* sexual transmission, direct *vs*. environmental transmission, or a simple *vs*. a complex life cycle. Third, and perhaps most importantly, is a growing ambiguity around the correct transmission metrics, the key measure of the parasite's fitness [14]. In particular, while transmission encompasses all the steps within and among hosts, many studies restrict themselves to a few of the steps (in particular, parasite load as a proxy for transmission), while overlooking the stage of transmission between two hosts (either biotic or abiotic) [14]. Factors, however, such as the host's dispersal [13, 15, 16] and the

parasite's ability to withstand environmental factors (e.g., temperature or dryness) [17–19] can affect the parasite's ability to infect a new host, and therefore our predictions regarding the spread and evolution of disease. A more inclusive framework of the factors that are likely to affect the transmission ability of a parasite has been developed [15]. This framework includes the whole life-cycle of the parasite, and in particular the parasite's intrinsic quality (which is called Q_p), linked to its ability to survive outside of a host, and the sensitivity of this quality to environmental factors (called Q_e).

In this study, we aimed to test aspects of this framework and examine whether it might help us understand the evolution of virulence and its relationship to transmission. We used the microsporidian parasite *Vavraia culicis*, which infects several species of mosquitoes [20]. It is transmitted through spores that are released into water by infected individuals and are then ingested by susceptible larvae to restart the parasite's life cycle [21]. Transmission is thus determined by a combination of the number of released spores and the longevity of the spores in the water. In particular, we used lines of the parasite that had been selected for early or late transmission [22] and that therefore had different levels of virulence. In our experiment we: i) characterized the ability of the lines to withstand the environment between hosts by measuring how their ability to infect a mosquito was affected by the time spent outside the host and by the temperature of their environment; ii) decomposed transmission according to the novel framework [15] and identified the components playing a role in the parasite's longevity in the environment; and iii) related the transmission components with their virulence. These steps, considering the time spent within hosts and in the external environment can help us to get a full picture of parasite evolution and reveal inherent trade-offs that might limit its evolvability.

Materials and Methods

Experimental model

We used the Kisumu strain of the mosquito host *Anopheles gambiae (s.s.)* and lines of the microsporidian parasite *Vavraia culicis floridensis* selected for seven generations: i) for early transmission and shorter time within the host or for late transmission and longer time within the host; ii) or lines taken from our unselected stock as a baseline reference [22]. Further details on the selection experiment and its maintenance are described in Silva and Koella (2024). Experiments and rearing were conducted at standard laboratory conditions (26 ± 1ºC, 70 ± 5% relative humidity and 12 h light/dark).

Preparation of parasite aliquots

Freshly hatched *A. gambiae* larvae were reared in groups of 50 per Petri dish (120 mm diameter x 17 mm) containing 80 ml of distilled water. They were fed daily with 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. Two days after hatching, larvae were exposed to 10,000 spores of *V. culicis* per larva, from spores obtained from one of the five lines of early-selected spores, one of the five lines of late-selected spores, or one of five replicates of the stock spore population. Upon pupation, individuals were transferred to cages (21 x 21 x 21cm) and left to emerge as adults. The adults were provided constant access to a 6% sucrose-water solution. Dead mosquitoes were collected shortly after their death until day 20 (when the experiment ended), and the remaining mosquitoes were collected. The collected mosquitoes were put into 2ml microcentrifuge tubes with 1ml of distilled water, with at most 25 mosquitoes per tube, and kept at 4ºC and in the dark. At the end of the collection, a stainless-steel bead (Ø 5 mm) was added to each of the tubes and the mosquitoes were homogenized with a Qiagen TissueLyser LT at a frequency of 30 Hz for two minutes. The resulting homogenate containing the parasite spores was transferred to a 15 ml falcon tube, and the spore concentration was estimated with a haemocytometer under a phase-contrast microscope (400x magnification). From each falcon tube we prepared aliquots with a spore concentration of 500,000 spores/ml for the infections in the following experiment. The resulting aliquots contained spores in 1:1 distilled water and an antibiotic-antimycotic cocktail solution (Sigma-Aldrich A5955, containing 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin per ml, filtered), which limits the growth of contaminating parasites without affecting microsporidia [23–26]. A preliminary experiment was run to ensure that the antibiotic-antimycotic solution did not interfere with the production of the parasite's spores or its development. Aliquots were kept in the dark at 4 ± 0.5 ºC or 20 ± 0.5 ºC dark until they were used.

Infectivity and infection severity experiment

To assay how time in the external environment and temperature of the environment affect infectivity, we infected mosquitoes (reared as described above) with one of the aliquots prepared 0, 45, or 90 days earlier. We used each aliquot only once to avoid contaminations. After pupation, mosquitoes were moved to individual 50 ml falcon tubes containing approximately 10 ml of distilled water [27], and at adult emergence, they were transferred to individual cups (5 cm Ø x 10 cm) with access to a 6% sugar solution and a humid filter paper. Ten days after emergence, the mosquitoes were frozen at -20ºC for later spore counts. At this time point, almost all of the mosquitoes infected with any of the parasite lines have started to produce spores [22].

Statistical analysis

The analyses were performed with R version 4.3.1 in RStudio version 3034.06.2+561. The packages "DHARMa" [28], "car" [29], "lme4" [30] and "emmeans" [31] were used to analyse the data.
Spore infectivity was assayed as the proportion of infected individuals. Hence, we assessed differences in infectivity using a generalized linear mixed model with a binomial error, and parasite

selection, time in the external environment, temperature and all interactions as explanatory variables. Selection line was included as a random factor.

Infection severity was defined as the spore load of individuals containing spores and was analysed with a linear mixed model with a Gaussian error and with the same explanatory variables and random factor as infectivity.

Following the framework developed by Silva et al (2024), we then quantified differences in spore quality (Q_p) and environmental effect (Q_e) for spore infectivity and infection severity [14] (Fig. 1). Q_p for infectivity was calculated as the difference within each selection line of infectivity (proportion of individuals with spores) at 0 days and 90 days (Fig. 2a), and Q_p for infection severity was calculated as the difference of spore load obtained after 0 days and after 90 days (Fig. 1b). As our standard environment we considered 4ºC, the temperature the spores have been adapted to for several years before this experiment. In both cases, we used a linear mixed model with parasite treatment as the explanatory variable and the replicate as a random factor.

Q_e for infectivity was calculated as the difference of infectivity between 4ºC and 20ºC for the time-point of 90 days, where we observe the most striking difference due to temperature (Fig. 2a). Q_e for infection severity was the difference of the average spore load obtained after 90 days between parasite kept at 4 ºC and 20ºC days (Fig. 1b). We analysed both using a linear mixed model with parasite treatment as the explanatory variable and the replicate as a random factor.

As a measure of virulence in the first host, we used the maximum hazards calculated by Silva and Koella (2024). We related virulence to infectivity by assessing whether the differences in Q_p or Q_e for infectivity or infection severity were explained by the virulence of each selection line. This was done with generalized linear models with quasi-Poisson error and where the dependent and the independent variables were transformed by adding 1 and taking the logarithm. The curves in Fig. 4a-d were calculated using the respective glm function with a Quasi-Poisson distribution.

Results

The direction of selection affected the parasite's ability to establish an infection in a new host, with stock and early-selected parasites being more infective than late-selected parasites ($\chi2 = 33.00$, $df = 2$, $p < 0.001$; Fig. 2a). The longer the spores were kept in the external environment, the less infective they were ($\chi2 = 77.56$, $df = 1$, $p < 0.001$), and if they were kept in the external environment at 20ºC they were less infective than if they were kept at 4ºC ($\chi2 = 166.85$, $df = 1$, $p < 0.001$; Fig. 2a). None of the interactions between selection treatment, temperature and duration in the external environment were significant ($\chi2 < 3.5$, $p > 0.05$). A similar pattern was observed for spore load (infection severity) of established infections (Fig. 2b) (selection: $\chi2 = 135.31$, $df = 2$, $p < 0.001$; duration in external environment: $\chi2 = 14.23$, $df = 2$, $p < 0.001$; temperature: $\chi2 = 4.46$; $df = 1$; $p = 0.035$). The effect of the duration in the external environment (interaction selection*duration: $\chi2 = 45.51$, $df = 2$, $p < 0.001$)

and of temperature (interaction selection*temperature: $\chi 2 = 7.35$; $df = 1$; $p = 0.025$) was more pronounced for parasites selected for late transmission than for early-selected or unselected parasites. For further details on the statistics, see SI Appendix Table S1.

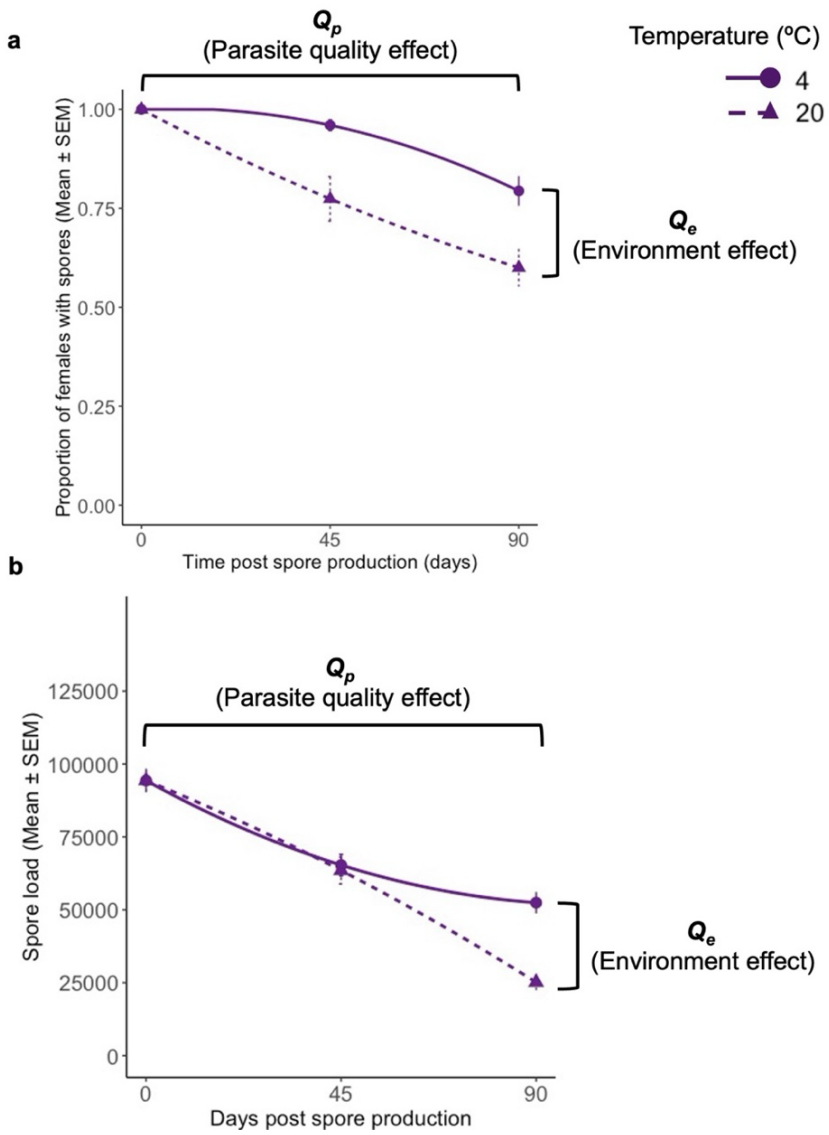

Figure 1. Illustration of Q_p and *Qe* calculation. Parasite quality (Q_p) and environment (Q_e) effect on infectivity (a) and infection severity (b) were estimated as demonstrated in the figure. In the figure, we demonstrate this rationale using the late-selected spores' infectivity and infection severity curves per environmental temperature and by time post-spore production presented in Figure 1. Q_p was measured as the mean difference in the proportion of females with spores/spore load between infection with 90-days and 0-days old spores at 4º C. Q_e, as the effect of environment temperature in this model, was calculated by subtracting the mean proportion of females with spores/spore load after exposure to spores kept at 20ºC for 90 days from the mean proportion of females with spores/spore load at 4ºC for 90 days. The resulting means of each replicate from each treatment were then used in Figures 3 and 4.

These analyses can be summarized with the two parameters Q_p (the difference in the standard environment (4ºC) between traits measured right after the host's death and after 90 days in the external environment) and Q_p (the difference of a trait measured after the spores had been kept for 90 days at 4ºC and 20ºC). Q_p for infectivity had a mean and standard error of 0.79 ± 0.04 in lines selected for early transmission comparatively to 0.93 ± 0.02 in lines selected for late transmission (Fig. 3a), while for infection severity a mean of 0.75 ± 0.09 in lines selected for early transmission and 0.51 ± 0.14 for late transmitted (Fig. 3a). Q_e ranged from 0.80 ± 0.06 (infectivity) and 0.78 ± 0.22 (spore load) in lines selected for early transmission to 0.76 ± 0.06 (infectivity) and 0.50 ± 0.16 (spore load) in lines for late transmission (Figs. 3cd).

The component of intrinsic quality, Q_p, was significantly linked to the average virulence of a selection line (Figs. 4ab), with more virulent lines being less able to infect a new host ($\chi2 = 13.00$; $df = 1$; $p < 0.001$) and producing fewer spores in the new host ($\chi2 = 4.58$; $df = 1$; $p = 0.032$) after a long time in external environment. The component of environmental sensitivity, Q_p, however, was not linked to virulence (Fig. 4cd). For further details on the statistics, see SI Appendix Table S2.

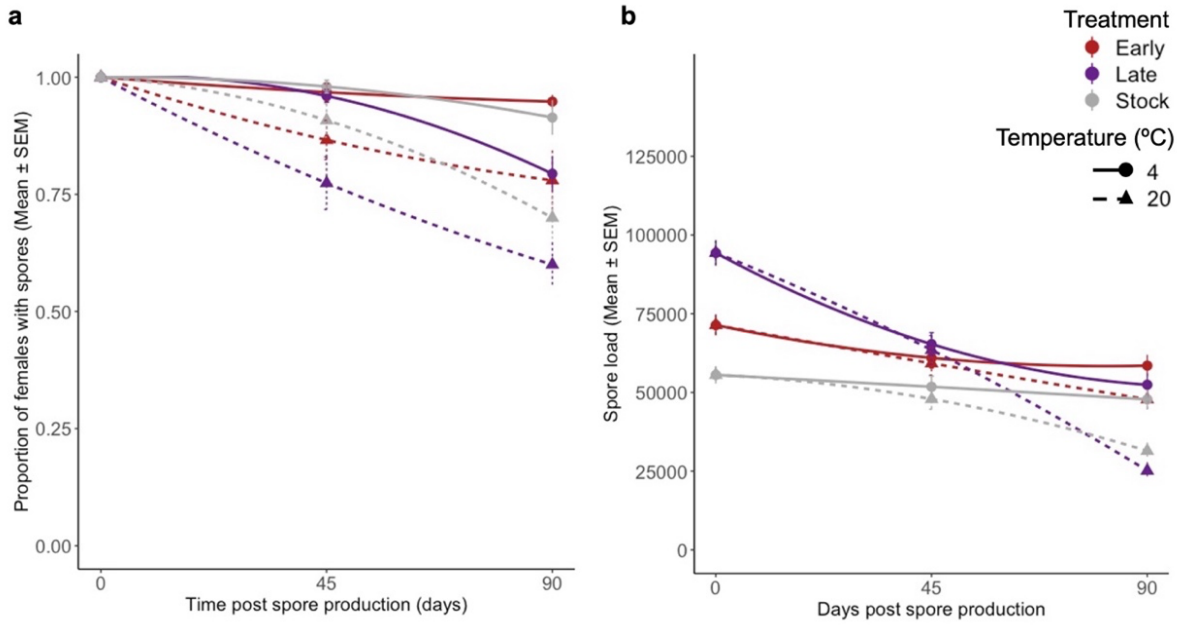

Figure 2. Parasite infectivity and infection severity in a new host. (a) Infectivity of the spores by regime, time and storage temperature. The latter was defined as the proportion of successful infections by day 10 of adulthood. Each mean consists of 150 females per treatment, time and temperature, split through five replicates. **(b)** Infection severity of the females carrying spores for the same time-point, as the number of number of spores per female. Sample size is the following for 4ºC and 20ºC, respectively. Day 0: All treatments consisted of 150 females. Day 45: Early with 145 and 130, Late with 144 and 116, Stock with 147 and 136. Day 90: Early 142 and 117, Late 119 and 90, Stock with 137 and 105.

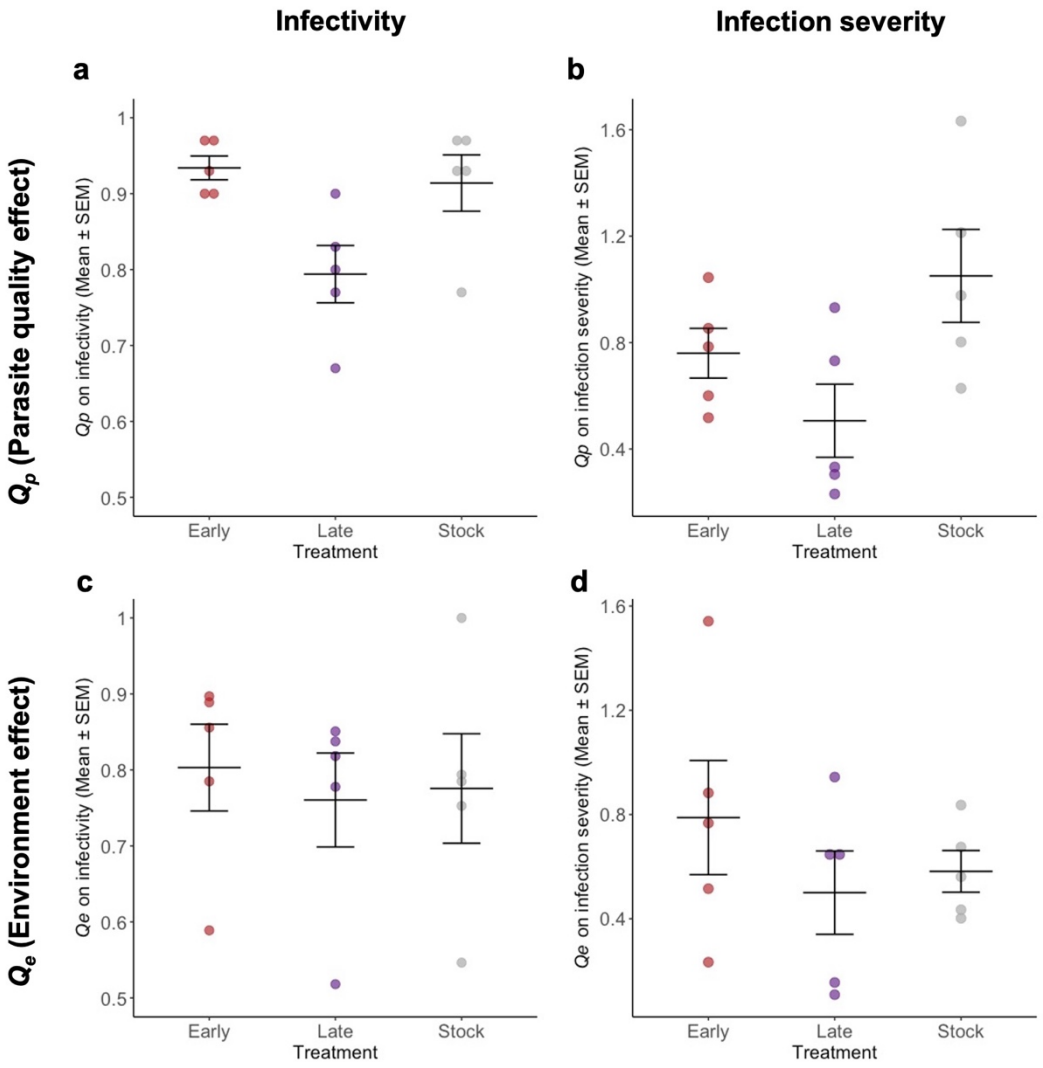

Figure 3. *Qp* and *Qe* for infectivity and infection severity. The effects of parasite selection on the parasite spore quality (Q_p) and environmental temperature (Q_e) for **(ac)** infectivity and **(bd)** infection severity (i.e., spore load). Each data point represents the mean value of one of the five replicated selection lines.

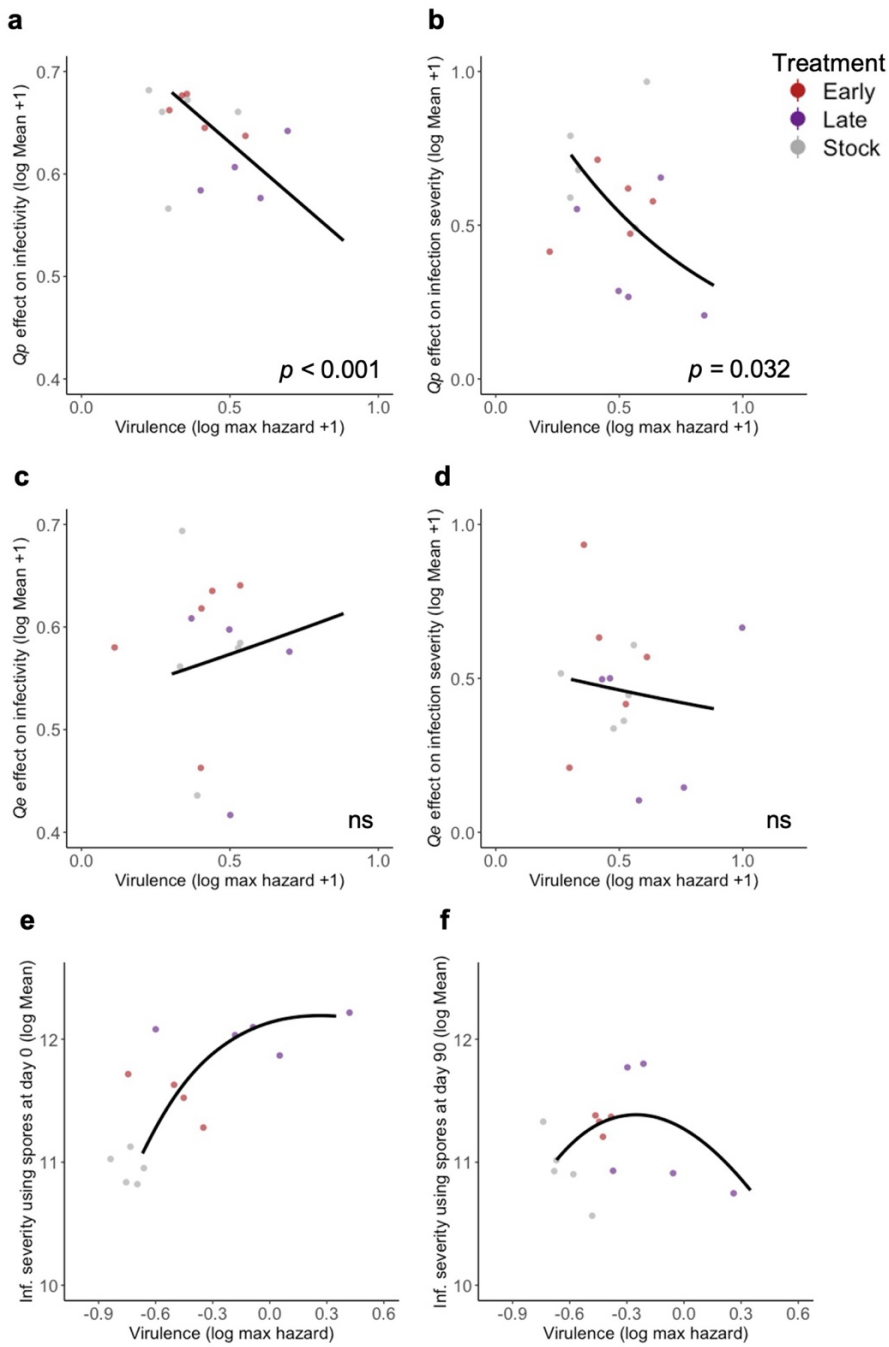

Figure 4. Virulence-transmission decomposition. Parasite's virulence in the first host in relationship to Q_p *and* Q_e effect on infectivity **(ac)** and infection severity **(bd)**, respectively. Each point represents the average values of a replicated selection line. The curves show the result of the general linear with a Quasi-Poisson distribution. Parasite burden in a new host caused after a time between hosts of 0 **(e)** and 90 days postproduction **(f)**. In these last two plots, a smooth line with the "loess" method was generated for ease of visualization.

Discussion

Many parasites spend time in an external environment as they transmit from one host to the next. While this phase of parasitic life cycles is often ignored in evolutionary ideas, we suggest that it may lead to important insights, in particular if the efficacy of transmission during the period spent outside of the host trades off with growth and virulence within the host. A standard prediction, for example, is that selecting for later transmission would lead to slower growth of the parasite within its host and lower virulence, while an experiment with lines of *V. culicis* selected for early or late transmission showed the opposite pattern [22]. This unexpected result could result from the fact that the experimental design eliminated any costs of virulence during the transmission phase in the external environment between hosts, which favoured the selection of parasites that invest in survival within the host rather than outside [22]. The results described here corroborate this idea.

First, the longer the spores were kept outside of a host, the less able they were to infect a new host (Fig. 1a). This result was particularly notable in the lines with the highest growth within the host (that is, the lines selected for late treatment), indicating a trade-off between growth and virulence within the host and survival outside of the host. Other studies have suggested a trade-off between virulence and survival either outside of the host or between hosts [32–34], and indeed adaptation to one environment (e.g., within the host) is generally expected to incur costs in other environments (e.g. outside of hosts). A similar pattern was found for infection severity (that is, spore load) in the next host (Fig. 1b), suggesting that the mortality of spores leading to less effective infectivity leads to a smaller dose of infection, and thus (as observed in other parasites) slower within-host growth later [35–37]. In addition, since infection severity decreases more rapidly than infectivity in the second time period in the external environment, time outside the host not only the survival of spores, but also their infection potential, which suggests that an additional process is operating. One possibility is that the resources within a spore may be consumed or degraded over time, which could delay its growth upon entering the host. Such a process, called growth arrest, has not been described for microsporidia but is common in bacteria [38, 39]. It helps the bacteria to meet their energetic and metabolic requirements before restarting replication, when cells become more susceptible to environmental stressors [40].

Second, the decrease of infection success with time spent outside the host was stronger when the spores were kept at 20ºC than when they were kept at 4ºC. Reduced spore survival at higher temperatures has been described for other aquatic microsporidia species, which thrive at lower temperatures [21]. In addition to their natural ecology, the parasite used in our experiment had been adapted to cold temperatures, for in our lab it is stored in the fridge. Again, this adaptation to one environment is expected to lead to lower efficacy in other environments, e.g., at 20 ºC, so the sensitivity to high temperature may well be higher in our experiment than in natural populations of the same parasite.

Third, by decomposing the parasite's transmission success across different selection lines and temperatures, we were able to disentangle the effect of environment (Q_e) and the parasite's intrinsic quality (Q_p) for infectivity and infection severity [14] and to describe how each component relates to virulence. While Q_e for neither infectivity nor infection severity was related to any of the treatments, Q_p was. Thus, late-selected spores were less resilient to time outside of the host compared to early-selected and control ones, but this resilience was intrinsic to the parasite and not affected by temperature (Fig. 3). Furthermore, Q_p, but not Q_e for both measures of transmission was negatively correlated with virulence (Fig. 4ab), suggesting a trade-off between virulence and parasite endurance in the external environment and corroborating that some microbes have a trade-off between reproduction and survival of their offspring generations.

Overall, our findings emphasize the importance of considering the whole transmission process in relationship to parasite and virulence evolution. Through the quantification of the parasite endurance to different environmental parameters, we were able to better explain the evolution of virulence in this system and we believe the same can be employed in many other taxa and infections. In fact, when we glance over the relationship between infection severity in a new host and virulence, we observe one of the advantages of having the full picture over transmission. While infection severity with freshly produced spores has a linear relationship with virulence (Fig. 4e), the same trait with 90-days old spores produces a concave shape (Fig. 4f), as expected from the virulence-transmission trade-off theory. Hence, we emphasize that this approach reflects the complex network between transmission and other parasite fitness traits, as well as the limitations and possible negative consequences of only looking at within-host parasite fitness. The study and dissection of this network and their fundamental trade-offs is essential to the understanding of parasite evolution, which is decisive when predicting disease spread and planning consequent control strategies. For instance, the findings in this study suggest certain social behaviours (e.g., crowding) or social settings might reduce the parasite time outside of the host and therefore inadvertently select for more virulent strains within the host.

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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Supplementary information

for " Virulence evolution: thinking outside of the host"

by Luís M. Silva and Jacob C. Koella

Table S1. Parasite infectivity and infection severity in a new host.

Tested effect			
Model 1a - Proportion of females with spores	**χ2**	***df***	***p***
Treatment	32.996	2	**< 0.001**
Temperature	77.557	1	**< 0.001**
Time	166.849	1	**< 0.001**
Treatment: Temperature	1.011	2	0.603
Treatment: Time	3.446	2	0.179
Temperature: Time	0.433	1	0.511
Treatment: Temperature: Time	1.867	2	0.393
Model 1b - Spore load	**χ2**	***df***	***p***
Treatment	135.307	2	**< 0.001**
Temperature	0.002	1	0.961
Time	14.228	1	**< 0.001**
Treatment: Temperature	0.814	2	0.666
Treatment: Time	45.506	2	**< 0.001**
Temperature: Time	4.459	1	**0.035**
Treatment: Temperature: Time	7.352	2	**0.025**

Table S2. Virulence-transmission decomposition.

Tested effect			
Model 4a - *Qp* on infectivity *vs.* virulence	**χ2**	***df***	***p***
log(Maximum hazard +1)	12.997	1	**< 0.001**
Model 4b - *Qp* on infection severity *vs.* virulence	**χ2**	***df***	***p***
log(Maximum hazard +1)	4.583	1	**0.032**
Model 4c - *Qe* on infectivity *vs.* virulence	**χ2**	***df***	***p***
log(Maximum hazard +1)	0.455	1	0.500
Model 4d - *Qe* on infection severity *vs.* virulence	**χ2**	***df***	***p***
log(Maximum hazard +1)	0.159	1	0.690