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Fungal response to abruptly or gradually delivered antifungal agent amphotericin B is growth stage dependent

Erqin Li [1,2]* Aleksandra Krsmanovic,[1,2]
Max-Bernhard Ballhausen [1,2] and
Matthias C. Rillig[1,2]
[1]*Institut für Biologie, Freie Universität Berlin, Berlin, Germany.*
[2]*Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), Berlin, Germany.*

Summary

Anthropogenic disturbances pose a multitude of novel challenges to ecosystems. While many experiments have tested effects using abrupt treatment applications, most environmental changes in fact are gradual. Since ecosystem responses might be highly dependent on the temporal nature of stressors, it is crucial to differentiate the effects of abrupt vs gradual treatment application. Antifungal agents, which are widely used in disease control both for humans and in agriculture, are becoming a new class of environmental contaminants. In this study, we examined the effect of a sub-lethal application of one antifungal agent, amphotericin B. We applied different rates of delivery, e.g. gradual and abrupt, and monitored biomass and sporulation of the model fungus *Neurospora crassa* in a batch culture. Our results demonstrate that: (i) the effect size difference between abrupt and gradual treatments is fungal growth stage dependent and (ii) the gradual treatment clearly had a higher sporulation level compared with all types of abrupt treatments. Our findings highlight the importance of considering the rate of change in environmental change research and point to a new research direction for future global change studies. Furthermore, our results also have important implications for avoiding treatment-induced spore production in agriculture and medical practise.

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Introduction

Anthropogenically induced environmental changes have become novel stressors to which most ecosystems inevitably are subjected. Over the recent decades, antifungal agents, which are widely used in disease control both for humans and in agriculture, have become a new class of environmental contaminants (Chen and Ying, 2015). After use, they enter wastewater, rivers, lakes and soil; in these environment compartments, antifungal agents pose unpredictable risks for both ecosystem and human health (Chambers *et al*., 2014; Assress *et al*., 2020). While the development of resistance during therapy or in horticulture has been addressed (Robbins *et al*., 2017; Revie *et al*., 2018), the ecological effects on non-target environmental species of environmental fungicide contaminants received little attention.

Amphotericin B (AmB) is one the most common agents to treat invasive fungal infections; it has been used for more than half a century in clinical treatment (Gallis *et al*., 1990). AmB is a broad-spectrum antifungal agent, and thus it is important to investigate its ecological non-target effects. The primary molecular target of AmB is the fungal cell-membrane lipid ergosterol. For decades, the idea has been that AmB binds with ergosterol, forming ion channel aggregates and thus increasing the permeability of the cell membrane, which in turn leads to the leakage of ions and in the end to cell death (Ermishkin *et al*., 1976). A more recent study shows that the cytocidal action of AmB is primarily through binding of ergosterol, and ion channel formation only serves as a complementary mechanism (Gray *et al*., 2012). A follow-up study reports that AmB primarily kills fungi by removing the vital lipid ergosterol from many aspects of cell physiology and forms extramembraneous and large fungicidal sterol sponges (Anderson *et al*., 2014). However, the most recent Raman scattering evidence once again supports the classical ion channel model instead of the newly proposed sterol sponge model (Dong *et al*., 2021).

Numerous current studies focus on the treatment efficiency of abruptly applied experimenter-determined levels of antifungal agents, but there is very little research on the temporal nature of exposure, most notably if the rate of change of antifungal agents could affect fungal responses. In fact, a lot of important and critical changes of environmental factors, including antifungal agents, change gradually rather than abruptly. It has been shown that different rates of change of environmental factors can lead to diverse ecosystem responses (Klironomos *et al*., 2005; Siteur *et al*., 2016). Since driver–response relationships are not necessarily constant through time but are modulated by the temporal characteristic of stresses (Ryo *et al*., 2019), it is important to examine the ecological effects of AmB on fungi at different rates of change.

Key aspects to consider when conducting experiments that vary the rate of change of environmental drivers are the magnitude of the treatment and the ramping time during which the stress is applied (Pinek *et al*., 2020). Intuitively straightforward as it might sound to set up comparable abrupt and gradual treatments, there are important points to consider. To reach the same magnitude, abrupt treatments would receive the dose of antifungal agent all at once with no ramping time, while the gradual treatment entails a longer phase for ramping up. It is thus inevitable to introduce differences in developmental stage or 'colony' ages of fungi and the perceived dose. A previous study from the laboratory, focused on copper as the stressor, used a dose-days approach ensuring the same area under the curve, which kept the incubation length and overall received dose the same for gradual and abrupt treatments (Golubeva *et al*., 2020). In order to achieve equivalent dose days with the gradual treatment, the abrupt treatment could either be harvested earlier or start later. Golubeva *et al*. (2020) started the abrupt treatment in the middle of the gradual treatment ramping period, ensuring the same dose days, when both treatments were harvested at the end of the gradual ramping. Hence, the growth stage of these two treatments was different when they encountered the added stress, and the gradual treatment always encountered a lower dose than the set magnitude until the end of the experiment and had shorter length of time under the maximum dose.

In the current study, we compared the effects of gradual and abrupt application of AmB with all the above concerns considered, by explicitly including different ways of delivering abrupt and gradual treatments settings. We compared the effect of abruptly or gradually applied sublethal AmB on *Neurospora crassa*, a common model strain in fungal biology (Davis and Perkins, 2002). The effect size was defined as the absolute biomass reduction compared with the control treatments with the same incubation length. This way of quantifying effect size allows us to compare treatments started and harvested at different time points and even ones with different incubation length. In order to test whether effect sizes of applied AmB are growth stage dependent, we included three abrupt treatments with different start time points in the current study. We hypothesize that abrupt treatments applied at an earlier fungal growth stage will have a larger effect size than those applied later, since a younger mycelium is generally believed to be more vulnerable to stressors than older mycelia (Dong *et al*., 2021). If this is true, then the comparisons of abrupt and gradual treatments with different starting points would be potentially misleading. In addition, we also include a gradual treatment that consists of a stasis phase, whose length is equal to the abrupt treatments. In such way, the gradual treatment has an equivalent number of days to develop responses under the same target concentration as the three abrupt treatments. We further hypothesize that abrupt treatments will generally have bigger effect sizes than gradual ones. Some studies have illustrated that organisms tend to deal with severe stress events better because of the priming effect, i.e., when a severe stressor is preceded by a milder one (a situation similar to but not entirely identical with gradually changing stressors) (Hilker *et al*., 2016). Finally, we hypothesize that the effect size differences between abrupt and gradual treatments will diminish over time due to the degradation/inactivation of antifungal agents in combination with compensatory growth.

Materials and methods

Experimental design

We used wild-type *N. crassa* (FGSC2489), a common model strain in fungal biology. The stock cultures were stored at −80°C in a 25% glycerol solution. The culture medium we used was Vogel's minimal medium (VMM) with 2% sucrose (Vogel, 1956). This medium is the standard culture medium for *N. crassa*, which contains all ingredients for optimal growth during the asexual lifecycle. Prior to the experiment, we inoculated FGSC2489 on VMM agar plates and incubated at 25° for 5 days. After 5 days, the asexual spores were harvested by adding 5 ml of DH_2O and vortexing for 15 s. This yielded a suspension with roughly 10^7 spores ml^{-1}.

In this study, 50 ml Mini Bioreactor tubes (Corning) were used to set up batch cultures, which contained 24 ml of VMM amended with 2% sucrose. Tubes were sterile and closed with a lid that allows for air exchange. We inoculated by adding 5 μl of the harvested spore suspension to the tubes. These cultures were incubated at 25°, 225 rpm shaking. Prior to the experiment, *N. crassa* dry biomass cultured in AmB free conditions was harvested daily ($n = 3$, day 0–10 and day 12), allowing us to predict the growth curve of *N. crassa* in our system.

Two days after the start of the incubation, allowing time for spore germination and fungal establishment, the AmB treatments were started (day 2). We used an AmB concentration of 0.1 μg ml^{-1}, which is a sublethal level for FGSC2489. Our preliminary test of minimum inhibitory concentration (MIC) of AmB for FGSC2489 showed that the MIC is 0.2 μg ml^{-1}. The inhibition test was performed with supplemented AmB ranging from 0.01 to 0.30 μg ml^{-1} in fivefold concentration steps. The test was initiated by inoculating a 96-well plate containing the series of AmB concentrations in VMM medium with spore suspensions, followed by incubation at room temperate (about 25°) for 48 h. Three replicates for each AmB concentration were used. Amphotericin B (Sigma-Aldrich) was first dissolved in Dimethyl sulfoxide (DMSO, Sigma-Aldrich) to make the stock solution with a concentration of 20 mg ml^{-1} and stored at 4°C. We took 24 μl of this AmB stock solution and dissolved it into a 14 ml of sterile culture medium as a substock. In order to reach the final concentration of 0.1 μg ml^{-1}, each tube received 70 μl of this substock solution. This volume is small enough to avoid nutrient supply differences between samples. In abrupt treatments, 70 μl of the substock solution were added at once, while in the gradual treatment, AmB substock solutions were added 10 μl day^{-1} for 7 days. The control treatments, with 70 μl Dimethyl sulfoxide (DMSO) adjusted AmB-free culture medium added, were incubated for the same number of days compared with each treatment, respectively. There were five replicates for all treatments. At end of the incubation, fungal dry biomass was determined. The mycelium was dried at 60°C till constant weight.

In order to test for any long-term effect difference between abrupt and gradual AmB treatments, in separate treatments, we extended the incubation times of the three different abrupt treatments and the corresponding gradual treatment to a longer incubation time after the final target concentration was reached (total of 27 days). After the incubation, we vortexed the tubes for 1 min for mixing. Then 200 μl of suspension was used to estimate spore yields with optical density (OD_{595}) followed by cell counting with a haemocytometer.

To illustrate the growth dynamics of *N. crassa* in our liquid culture system, we detected the daily growth for 12 days and fitted the data to a logistic growth model (Fig. 1A, black line). Since the fungal growth rate in this system is not constant over time, the real-time growth rate was predicted as the first derivative of the best-fitted logistic growth model (Fig. 1A, blue line). According to the observed data and predicted model, the lag time is about 1 day, the exponential growth phase is from days 2 to 8, and from day 9 on the fungal biomass stays in a stationary phase.

Statistics

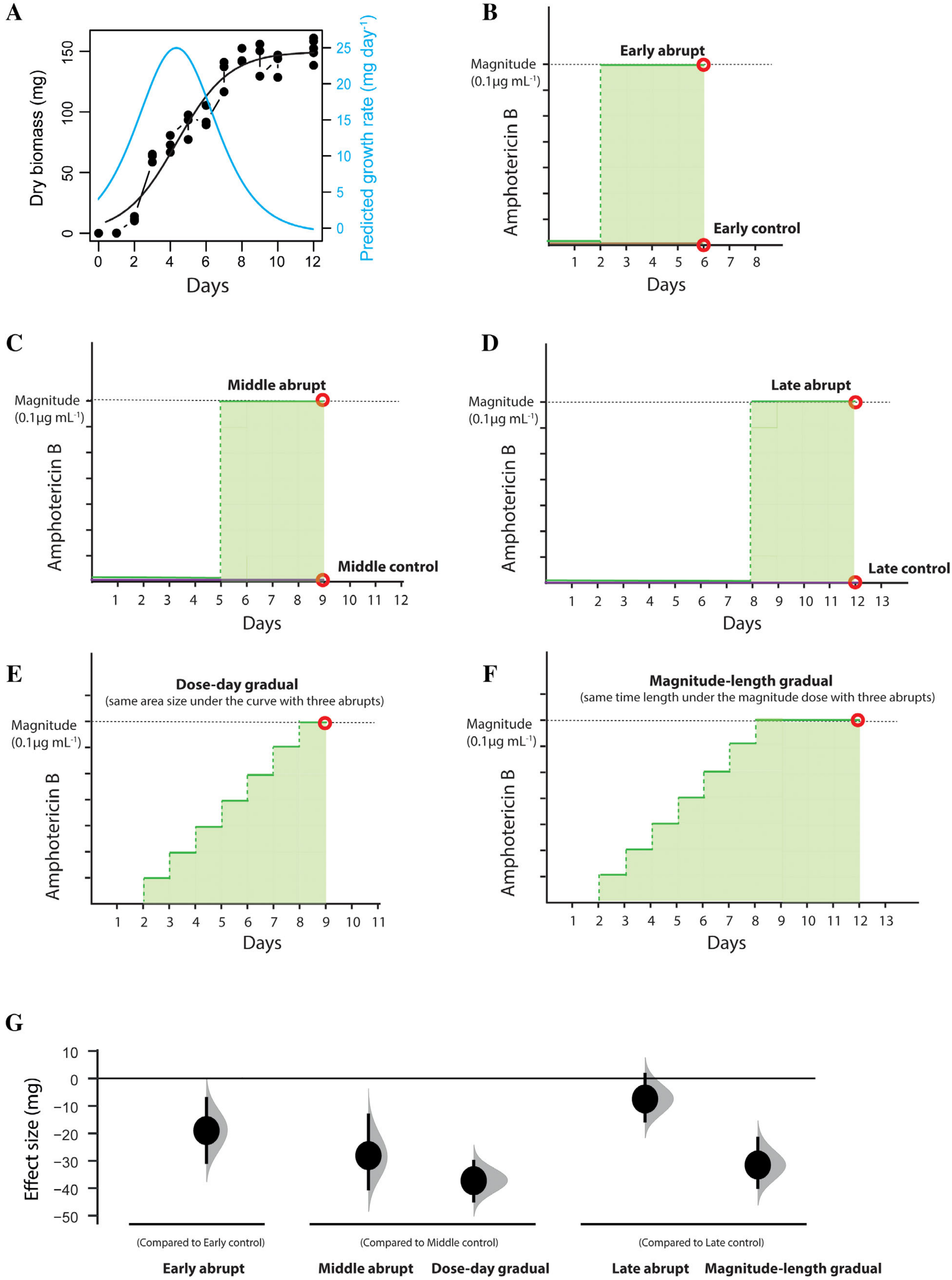
The effects of treatments were quantified by the effect size (mean difference) compared to their corresponding control treatments. The effect size, and its 95% confidence interval (95% CI) were computed through 5000 times (5000 resampling, 5000 replicates), nonparametric, bias-corrected and accelerated (BCa) bootstrap as default setting with *mean_diff*() function from R package ‘dabestr’ (R code and data are available in Supporting information).

Results

Short-term abrupt vs gradual treatments

Based on our growth curve model, three 4-day long abrupt treatments were applied at early, middle and late growth stages respectively. An early abrupt was applied on day 2, which is the beginning of the exponential growth phase, here named ‘early-stage abrupt’ (Fig. 1B). A second abrupt treatment was applied on day 5, in the middle of the exponential growth phase with the highest predicted growth rate, named ‘mid-stage abrupt’ (Fig. 1C). The third abrupt treatment (‘late-stage abrupt’) was applied at the end of the exponential growth phase on day 8, when the growth rate is expected to be zero (Fig. 1D). Two gradual treatments consist of the same ramping stage covering the whole exponential growth phase from days 2 to 8, and we distinguished ‘dose-day gradual’ and ‘magnitude-length gradual’ treatments. The ‘dose-day gradual’ treatment has the same dose days, e.g. area under the curve, compared with all the three abrupt treatments (Fig. 1E), while the treatment ‘magnitude-length gradual’ experienced the same length of time under the target AmB concentration as the abrupt treatments (Fig. 1F).

Biomass reductions, that is, negative effect sizes, were found in all but one abrupt and gradual treatments (Fig. 1G). Early abrupt caused 19 mg (mean; ci_low −31.1, ci_high −6.74) biomass reduction on average. Middle abrupt led to the largest biomass reduction among all abrupt treatments, 28.1 mg (mean; ci_low −40.7, ci_high −12.76) on average. The Dose-day gradual treatment led to the largest biomass reduction on average, 37.3 mg (mean; ci_low −45.1, ci_high −29.62). The magnitude-length gradual treatment led to a similar biomass reduction, 31.54 mg (mean; ci_low −40.20 ci_high −21.30) with dose-day gradual and middle abrupt. The 95% CI overlapped with the zero line (line of no effect) in the late abrupt treatment, which indicates a neutral effect.


A
Dry biomass (mg)
Predicted growth rate (mg day^{-1})
Days
B
Early abrupt
Magnitude
(0.1μg mL^{-1})
Amphotericin B
Early control
Days
C
Middle abrupt
Middle control
D
Late abrupt
Late control
E
Dose-day gradual
(same area size under the curve with three abrupts)
F
Magnitude-length gradual
(same time length under the magnitude dose with three abrupts)
G
Effect size (mg)
(Compared to Early control)
(Compared to Middle control)
(Compared to Late control)
Early abrupt
Middle abrupt
Dose-day gradual
Late abrupt
Magnitude-length gradual

Abrupt vs gradual treatments in long term

To verify the long-term effect size difference between gradually or abruptly applied antifungal agents, we extended the experimental length to 27 days, which means that the time in the stationary phase is about 18 days. After this long culture under stationary-phase nutrients are likely becoming limiting in batch culture. The long-term gradual treatment consists of a 7-day ramping phase followed by 18-days incubation in the stationary phase (long gradual, Fig. 2A). Three abrupt treatments were applied at early, middle and late stages of the exponential phase, named long-early abrupt, long-middle abrupt, long-late abrupt respectively (Fig. 2A). The biomass at day 27 dropped more than 40%, (mean, 86.58 mg) compared with the maximal biomass K, predicted by the growth model in Fig. 1A. Although our design could not record the decline dynamic, it is clear that there is a biomass decline after the stationary phase.

After longer-term incubation, the long early abrupt treatment resulted in a neutral effect (Fig. 2B). The long late abrupt treatment was consistent with the late abrupt, which also resulted in a neutral effect. The long middle abrupt was the only abrupt treatment that produced a negative effect on fungal biomass (mean, −6.48 mg; ci_low −11.68, ci_high −2.46). In general, the negative effect on fungal biomass in all treatments was smaller compared with the earlier harvest (Figs. 1G and 2B). Long-gradual led to a clear negative effect on fungal biomass (mean, −11 mg; ci_low −16.8, ci_high −2.99), and it is not clearly different from the effect size of long middle abrupt.

Fungal sporulation was evident in all abrupt and gradual AmB treatments (Fig. 2C). In contrast, there was no sporulation in the AmB-free control. The gradual treatment led to the highest sporulation both in total amount of spores (Fig. 2D) and sporulation per gram of biomass (Fig. 2E) compared with abrupt treatments. Surprisingly, the long early abrupt treatment, which was exposed to antifungal agent the earliest and longest, resulted in the lowest sporulation.

Discussion

In our system, at the organism level, the effect size of AmB is largely dependent on the growth stage of *N. crassa*. We found that early and middle abrupt showed a clear negative effect (effect size below 0), while late abrupt surprisingly showed a neutral effect, suggesting that this abrupt treatment did not influence the growth of fungi (Fig. 1G). One might suspect that larger resident biomass present during later growth stages would reduce the dose of antifungal agent per gram of fungal biomass, thus resulting in attenuated effects. However, the effect size in early abrupt is not larger than in the middle abrupt treatment, where the resident biomass is much smaller (Fig. 1A, black line). A more plausible reason might be that the growth stage dependency of effects reflects different affinity of fungal cells to AmB as we have hypothesized. Binding to the fungal cell wall is an initial step for this antibiotic agent to affect the fungal cells. It has been reported that the thickening of the cell wall plays an essential role in preventing AmB insertion into the membrane, and the stationary-phase fungal culture exhibits two or three times greater magnitudes of resistance to AmB compared with the log-phase one (Gale *et al*., 1980). Supporting this, recent visual evidence by Raman scattering imaging resolved that the metabolically active log-phase exhibits higher affinity to AmB compared with cells in the stationary phase (Dong *et al*. 2021). This indicates that the stress–response relationships are not necessarily constant through time but conditioned by the growth stage of the organism, in our case the developing fungal individual. Thus, we conclude that the temporal character of stress application is highly relevant for the outcomes of the effect size and should be carefully considered in experimental designs addressing temporal dynamics of stressor effects.

Dose-day gradual showed no difference compared to two log phase applied abrupt treatments (middle and late abrupt), but its effect size is clearly different from late abrupt (Fig. 1G). Thus, it is important to consider the

Fig. 1. A. Growth curve of *Neurospora crassa* (FGSC2489) in 24 ml batch culture. The x-axis shows the incubation days, and the y-axis shows the dry biomass (mg) of fungi ($n = 3$ day^{-1}. exception on day 12 $n = 5$). The black line represents the logistic model $N(t) = K/(1 + ((K - N0)/N0) * \exp(-r * t))$ fitted to the growth curve, with maximum biomass $K = 149.7$ mg, initial biomass $N0 = 7.2$ mg, and specific growth rate $r = 0.69$. The growth curve model was estimated with the R package '*growthcurve*'. The real-time growth rate of *N. crassa* was predicted as the first derivative of the fitted growth curve logistic model (blue line).
B–F. Experimental settings. The x-axis represents the incubation days. The fungal incubation started on day 0. The red circle indicates the harvest time point. The brown lines next to the x-axis indicate the AmB-free control. The y-axis represents the concentration of AmB. The magnitude of the aimed AmB concentration is 0.1 μg ml^{-1}, which is indicated by the dashed line. The green lines indicates the concentration of AmB over time. The size of the areas under the curves, filled with light green, is equivalent among dose-day gradual and all abrupt treatments. The incubation lengths under the target AmB concentration of magnitude-length gradual and all abrupt treatments are the same.
G. The effect size of abrupt and gradual treatments. The effect size was defined as the biomass difference of all treatments compared to AmB-free controls. Circle represents the bootstrapped effect size mean (effect magnitude) and vertical line the corresponding 95% confidence interval (effect precision). The density plots depict bootstrapped data distribution.

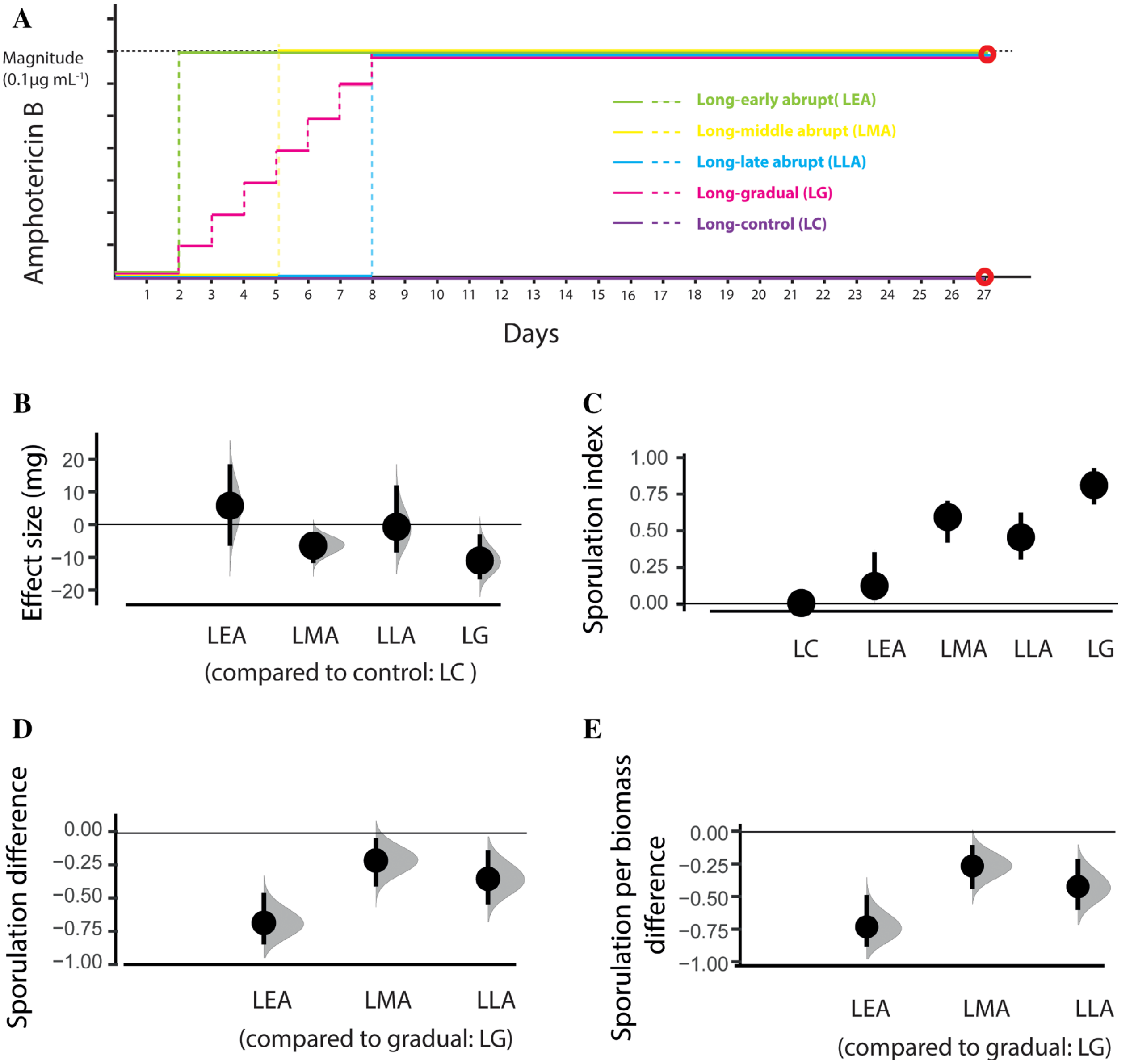


Fig. 2. Long-term effects of abrupt and gradual AmB treatments.
A.. Experimental settings. The x-axis represents the incubation days. The red circle indicates the harvest time point. The purple line on the x-axis indicates the AmB-free control. The y-axis represents the concentration of AmB. The magnitude of the target AmB concentration is 0.1 μg ml^{-1}, which is indicated by the dashed line. The different coloured lines indicate the concentration of AmB over time.
B. The effect size of the three abrupt and the gradual treatments compared with their AmB-free control treatment. The effect size is calculated based on fungal dry biomass.
C. Fungal sporulation recorded for the three abrupt and gradual treatments and the AmB-free control treatment. The sporulation index ranges between 0 and 1, with 0 indicating zero sporulation.
D. The difference of sporulation between gradual and abrupt treatments.
E. The difference of sporulation per biomass between gradual and abrupt treatments. Sporulation per biomass is defined as sporulation per gram of fungal dry biomass. The sporulation per biomass was normalized between 0 and 1, with 0 indicating spore free. In panels B–E, the treatments abbreviation are the same as indicated in panel a: LC, long control; LEA, long-early abrupt; LMA, long-middle abrupt; LLA, long-late abrupt; LG, long-gradual. The circle represents the bootstrapped mean and vertical line the 95% confidence interval. The density plots depict bootstrapped data distribution.

growth stage of an organism, e.g. a growing stage or stationary stage, when setting up an experiment to compare the effect of gradually and abruptly delivered stress, especially when the gradual application is long enough to cover different organism growth stages. In this case, deriving a conclusion from a comparison with one particular abrupt treatment applied at a certain time point can be misleading. We believe that this conclusion is also valuable when considering other antifungals despite of their distinct mode of actions. For example the application of azoles, which block the ergosterol biosynthesis (Robbins *et al*., 2017), could lead to ergosterol shortage in actively growing cells and show growth stage-dependent effects.

The magnitude-length gradual treatment, which was extended for 3 incubation days compared with dose-day gradual, showed a similar effect size to dose-day gradual. Based on the growth curve in Fig. 1A, the end of the ramping phase is also the end of the fungal log growth phase, if we assumed that the gradual AmB addition did not drastically change the growth curve of *N. crassa*. Together with the neutral effect of late abrupt, we conclude that negative effects are rare during the stationary phase even when reaching the magnitude of the final target AmB concentration. In this case, it does not make much sense to ignore the ramping period and compare the effects of gradual and abrupt treatments with the same response length under the final stressor magnitude; that is, we do not recommend further use of this 'magnitude-length gradual' treatments under such conditions.

In the longer-term experiment, the gradual treatment continued to show a negative effect over time, although with reduced magnitude; this suggests that the persistence of such effects when the stressor was delivered gradually. In the three abrupt treatments, however, only for the middle abrupt was there a negative effect, while both early and middle abrupt had neutral effects. This is largely in line with our hypothesis that states AmB treatment effect size reduction over time. Interestingly, this also suggests that abrupt treatments' negative effect is of shorter duration (as in early abrupt) than observed in the gradual treatment. The differences in effect among the different abrupt treatments might also indicate that the duration of effect persistence might depend on the fungal growth rate at the moment of AmB application: when applied at the highest growth rate, the antifungal agent resulted in the longest-lasting effect.

Abrupt treatments have not shown bigger effect sizes than gradual ones neither in short-term nor long-term based on fungal biomass data. In the contrast, there is a clear difference between gradual and abrupt treatments in fungal sporulation, in that the former induced a larger amount of spores than all abrupt treatments at the end of the long-term incubation. This trend is also true when we consider sporulation expressed on a per biomass basis. Fungal spores are considered as a means of stress-resistance/avoidance in space and time (Dijksterhuis, 2019). Spatial resistance means that fungal spores can leave the local conditions and travel by a range of different means, e.g. air, towards new habitats to colonize (Damialis *et al*., 2017). Temporal resistance means that the stabilized cells, spores, enable fungi to survive at their local position waiting for better conditions to germinate (Dijksterhuis, 2019). First of all, it is inevitable to experience deprivation of nutrients in batch culture. In general, the nutrient deprivation leads to the formation of aerial hyphae, at the end of which budding structures (conidiophores) are formed that generate vegetative spores, the macroconidia. Since we did not detect sporulation in the control treatment, where the fungal biomass was the highest and should experience starvation earlier and more severely, it is not likely that the difference in starvation timing and severity among treatments is the main reason for sporulation differences. Desiccation, lack of water, is another reason that leads to differentiation of aerial hyphae from basal hyphae, but it is not likely playing a role in our liquid culture. Although we did not detect spores in control treatment after about 1 month, all AmB treatments, regardless of the abrupt and gradual, successfully induced sporulation. Thus, we conclude that it is the antifungal agent application that induced the sporulation and the ways of AmB delivery resulted in the differences in sporulation. It might be a result of evolutionary selection that under stress conditions forming large numbers of spores could increase the survival rate both because of large population size and higher resistance to environmental stressors in a dormancy status (Zhang *et al*., 2015).

The reason why the gradual treatment induced larger sporulation rate than the abrupt treatments is unclear. Since our abrupt treatments covered the early, middle and late growth stage, we can exclude the possibility that it is because of the moment of application. Early abrupt treatments resulted in a very low amount of spores, while the two other abrupt treatments showed much larger sporulation rate. This suggests that timing of antifungal agent application makes a large difference; meaning that AmB applied during later growth stages is more likely to induce sporulation. As early abrupt and gradual have the same exposure time to AmB, we can also exclude the possibility that longer exposure could induce more fungal sporulation. We also know that growth rate and asexual reproduction in *N. crassa* are highly independent, and that there is not an apparent trade-off between these two traits (Anderson *et al*., 2019). Therefore, growth rate changes in response to AmB application may not be the underlying cause. Possibly, Amb application might have induced differences in sporulation regulatory gene expression. For example, there are genes that control the length of aerial hyphae (Colot *et al*., 2006), which could be a crucial trait for successful sporulation in our experimental system. A promising future research direction would be to compare the gene expression pattern differences between abrupt and gradual treatments, which would give an overview of differently affected pathways and metabolic processes.

Our study suggests that naturally occurring environmental changes should be simulated as gradually changing factors since the traditional abrupt treatment delivery could lead to underestimation of their effects. Also, it is essential to consider different fungal life stages, e.g. vegetative growth and sporulation stage since fungal growth stage will affect treatment perception. As we show here, fungal biomass and sporulation showed a completely different trend among treatments. In particular, the induction of sporulation in all treatments suggested that applied AmB had caused important physiological changes in fungi, which may not be reflected in biomass differences. The highest sporulation under gradually delivered stress is an alarming reminder that in reality anthropogenic disturbances might have much severe consequences than expected. The large sporulation output we observed, representing a large number of dispersal-stabilized cells, could pose problems either in terms of efficiency of disease control or for ecosystem stability, for example, since this increased spore output could increase the risk of invasion events.

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Author contributions

E.L. conducted the study, which was designed by E.L. and M.C.R. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information