Research Design

Minerva University

NS51: Empirical Analyses

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Cover sheet

Questions about your study design	Your response
What is your research question?	Is heat exposure associated with fungi's acquired thermoresistance?
Write two hypotheses and put the one that you will focus on in your paper in bold.	- Fungi's increasing acquired thermoresistance is associated to heat-shock protein production (Tiwari, S. et al, 2015) - **Fungi's increasing acquired thermoresistance is associated to the heat stress-induced mutations**
For your bolded hypothesis that you will focus on in your paper, write three predictions. Choose one of those predictions to focus on in your paper and put it in bold. Your study design should propose how to test this prediction to determine support for or against your bolded hypothesis.	- Fungi sampled from high-temperature environments will present higher growth rates than those sampled from low-temperature environments - Giving the rising temperature trends caused by the climate crisis, later generations of fungi will present a higher base thermoresistance

	- Fungi that have evolved in environments with higher average temperatures will have a higher baseline thermotolerance than those from cooler environments.
What are potential ethical considerations of your study?	- A salient ethical consideration for this study would be "in what environment/host would this experiment measure the growth or evolution of the sample fungi? Are living subjects/hosts going to be used?" - A potential side-effect of this experiment can be creating thermoresistant colonies of fungi, which if not disposed appropriately could negatively impact a local ecosystem by introducing an invasive species. - Researchers' interests might cause them to steer the results or conclusions of the study so that they align with

	their interests.
What type of research design will you use?	Quasi-experimental
What sampling strategy will you use?	Convenient and purposeful
What are your treatment(s)	Exposure to different temperatures
What are your independent and dependent variables?	Independent: temperature Dependent: thermoresistance, measured by growth rate
What are your controls?	pH
What are potential confounding variable(s)?	Protein/nutrient availability, humidity, pH, carbon concentration
Does your study design properly address ethical considerations?	- No living subjects/hosts are used in the experiment. Instead, inspired by a past study conducted on bacterias, in-vitro colonies of fungi are grown. - The study proposes the usage of an antifungal agent to safely dispose of the products of the experiments. However, just as bacterias can develop resistance, fungi can develop resistance to antifungal agents; more

	research is needed to determine the effectiveness of this measure. - To account for the researchers' potential confirmation bias, all the parameters for the experiment are defined ahead-of-time, even before sampling the fungi populations to be studied and, once sampled, blinding is applied.

Introduction

The kingdom of fungi presents a great variety that can be classified based on morphology, growth and development, reproduction, evolution, infection-causing ability, and toxigenicity, among others. However, most fungi share a common property - their biological inability to survive at high temperatures (Fisher, M. C. et al, 2020). This limits the range of biological agents that can be affected by fungi, reducing the hosts to cold-blooded life forms.

The climate crisis has caused global temperatures to rise, and it is projected to continue at an accelerated rate. Some studies have shown that in areas of the U.S. particularly affected by the climate crisis, fungi have acquired increased thermoresistance (i.e., the ability to withstand higher temperatures) (Byrnes III et al, 2010). On the topic of "How do humans impact

evolution?", this paper proposes an experimental design to explore the potential association between heat exposure and fungi's acquired thermoresistance.

Fungi's increased heat resistance might be caused by high production of heat-shock proteins (Tiwari, S. et al, 2015) (i.e., proteins that guide certain biological functions) (adaptative) or increased mutation rates caused by heat stress (Gusa, A. et al, 2023) (evolutionary); more confounding causes can be related to pH levels, humidity and carbon concentration.

This paper focuses on the evolutionary hypothesis, examining the evolutionary impact of heat stress. Specifically, given the ongoing climate crisis, the proposed experimental design aims to provide evidence supporting the prediction that later generations of fungi will present higher base thermoresistance which could lead to an increased infection rate for hot-blooded life forms.

Research design

Research has shown that environmental stressors can cause DNA elements, like transposons, to move within the genome. For example, a study found that heat stress can cause DNA mobility and lead to rapid changes in the fungal pathogen Cryptococcus (Gusa, A. et al, 2023). Genetic transposition, the same mutation mechanism, can create large-effect mutations that help with rapid adaptation (Quadrana, L. et al, 2019).

In this section, this paper presents an experimental design to provide evidence for the potential association between fungi's increased acquired thermoresistance and heat stress-induced mutations. The design will examine the relationship between environmental temperature, measured in degrees Celsius (independent variable; quantitative continuous, but for

the purposes of this study, treated in discrete intervals), and thermoresistance, measured as growth rate (dependent variable; quantitative discrete)[1][2][3].

Measuring thermoresistance in fungi is difficult due to the lack of a clear and affordable method in academic literature. This report suggests using growth rate as a measure of thermoresistance. Microorganisms with low thermoresistance have a low growth rate when exposed to high temperatures, while microorganisms with high thermoresistance demonstrate steady growth rates regardless. The growth-rate measured by the number of microorganisms in a given colony, which can be estimated by measuring the colony's physical dimensions (area in cm^2) multiplied by the density of microorganisms (organisms / cm^2)[4].

Four factors may affect the phenomenon of fungi's increased thermoresistance: protein/nutrient availability, humidity, pH, and carbon concentration. Higher nutrient and protein availability might increase the production of heat-absorbing proteins. High humidity

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[1] **#evidencebased**: The applications of this HC follows two main peer-reviewed lines of evidence: (a) heat stress increases transposon mobility (i.e., genetic mutations) and (b) transposon mobility leads to large-effect mutations that increase the organism's rapid adaptation. This paper builds on top of these two premises to propose an experimental setting for studying the impact on heat stress on fungi's acquired thermoresistance.

[2] **#hypothesisdevelopment**: The application of this HC tackles the phenomenon of fungi's acquired thermoresistance and states several plausible explanations (i.e., heat-shock protein production, and heat stress-induced mutations). Thereby, creating a testable hypothesis, deriving predictions from it, and proposing an experimental setting to provide evidence for the hypothesis and giving insight into the predictions.

[3] **#plausibility**: The application of this HC bases the proposed and revised hypothesis on peer-reviewed evidence proposed by two different studies. The conclusions of both studies used as premises –as well as all the other sources cited throughout this paper– seem to support, or at the very least depict as plausible, the proposed hypothesis. In particular, systematically exposing fungi to heat would lead them to experience increased transposon mobility (i.e., higher mutation rates), which as seen in Baym, M. et al (2016), can lead organisms to develop resistance to environmental stress –in this case heat.

[4] **#variables**: The application of this HC identifies and defines all the relevant variables involved in the hypothesis. Furthermore, it recognize nuances for both of the variables; in the case of the independent variable, acknowledges that for the purposes of this study, the variable is going to be treated as discrete; for the dependent variable (thermoresistance), a proxy variable is used since there's no cost-effective way of measuring thermoresistance for fungi. The procedure of how to compute the dependent variable is detailed and explained.

environments might help heat spread, whereas low humidity environments might increase the survival rate of fungi exposed to high temperatures. Environments with lower acidity may also explain the higher survival rate for fungi exposed to high temperatures. Lastly, higher carbon concentration environments may facilitate fungal growth and thus explain the higher survival rate for fungi exposed to high temperatures.

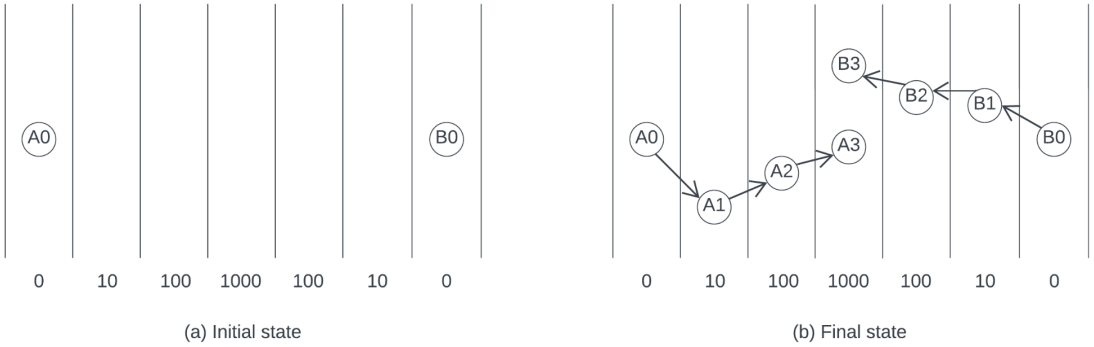

Figure 1: *Illustration of the experimental setting conducted by Baym, M. et al (2016). The experiment proposed growing colonies of bacterias on both ends of a large petri dish. The initial colonies were put in a zone with 0 concentration of antibiotics (a). As the colonies grow, they encounter bands of increased antibiotic concentration (by a factor of 10). As time passes, the colonies mutate and acquire antibiotic resistance. After 2 weeks, the bacterial populations colonized the entire petri dish (b). The trajectories shown in the figure are only illustrative; the real trajectories can be found in Baym, M. et al (2016).*

The proposed design takes inspiration from a study conducted by (Baym, M. et al, 2016). In this study, the researchers designed a setting that visualized the evolution of bacteria when exposed to environmental stress (see Figure 1). The stress was caused by increasing concentrations of antibiotics in discrete exponential steps, starting from a concentration of 0, then 10^1, 10^2 and 10^3.

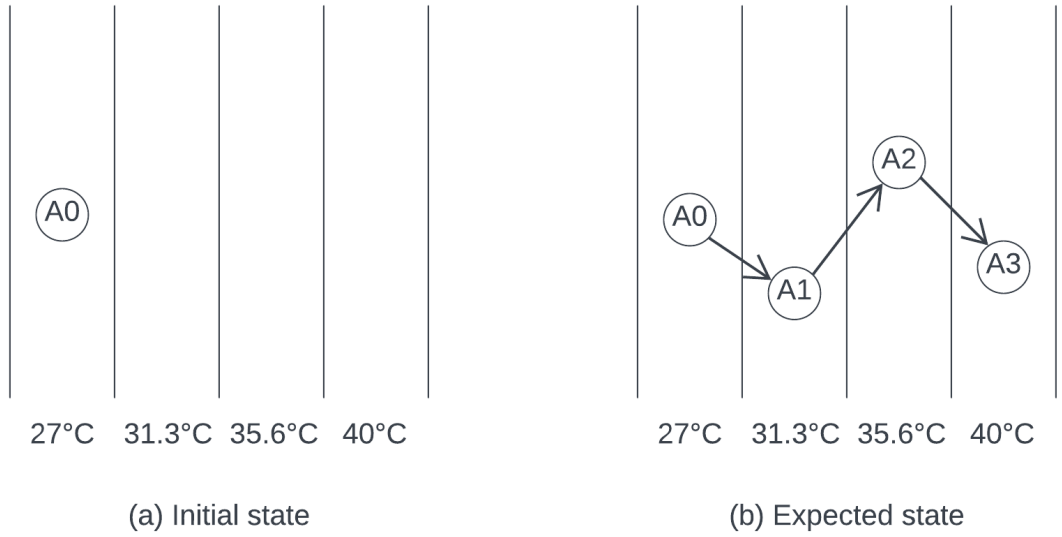

Figure 2: *Illustration of an experimental unit of the proposed experimental setting. The fungi colonies are initially put in a state with their natural environmental temperature at a controlled pH and nutrient/protein level (uniform). (a) It is expected that the fungi experience heat stress, which would lead them to mutate and acquire thermoresistance, therefore colonizing the entire petri dish (b).*

In the proposed design, instead of using a gradient of antibiotic concentration, a temperature gradient is proposed to represent a natural environmental stress (climate crisis) (see Figure 2). This will allow for the evaluation of how the growth rate of a fungus changes with temperature and perform comparative pre-tests and post-tests[5].

To evaluate the growth performance of the intervened colonies, the resulting colonies are sampled and grown again in an environment with a higher initial temperature. This will help to measure the growth performance of the colony after it has been subjected to the temperature

[5] **#testability**: The application of this HC proposes pre-tests and post-test to measure the effectiveness of the proposed intervention. These tests directly relate back to the stated predictions that derive from the hypothesis. Furthermore, the study procedure is clearly stated and outlined, presenting a relatively low-cost allowing for replication to further validation of the results.

gradient (post-test). The colony will be monitored to determine if it has adapted to the new temperature and how it performs compared to a control group that was not subjected to the temperature gradient.

The objective of this experiment is to establish a causal relationship between variables. However, due to the vast number of fungi species available, it is impractical to randomly sample from all of them. Therefore, a quasi-experimental[6] design is proposed that uses convenient and purposeful sampling by surveying fungi species that are most accessible in urban contexts, such as murine-carried fungi, mold, etc. (i.e., most plausible sources of infections for Humans). This approach mixes between convenient and purposeful sampling, since the samples are comparatively easy to obtain and they would give us insights into realistic potential pathogens for Humans[7].

To further improve the design, the setting takes into account pH as a possible confounding variable. It generates groups with different base pH levels to ensure that any potential changes in the dependent variable (i.e., thermoresistance) are not due to differences in pH levels. By doing this, this design will be able to obtain accurate and reliable results.

The proposed procedure goes as follows:

1. Set a timeframe (i.e., 3 weeks), maximum temperature (i.e., 40 °C), pH levels to tests (i.e., 6, 7, and 8).

[6] **#interventionalstudy**: The application of this HC proposes an interventional study to test the hypothesis. The sampling mechanism used for this study is convenient and purposeful, since random sampling would be infeasible due to the great diversity among fungi species. The study is described in detail, clearly stating the control groups and the interventions as well as the proposed confounding variables to be controlled. Further improvements for the study are proposed.

[7] **#sampling**: The application of this HC explains why convenient and purposeful sampling methods are chosen. Random sampling is not feasible given the vast diversity in fungi species. Advocates for more plausible pathogen agents as a justification for the convenient sampling.

2. Sample fungi species.
3. Anonymize and shuffle the samples (blinding).
4. For each sampled specie:
    1. Determine the natural environment's temperature.
    2. Create a **Natural History group (control group)**, only exposed to its natural environment's temperature at a base pH level (**pre-test**).
    3. For each established pH level:
        1. Create a **Positive Gradient of Temperature group (treatment group)**[8], from its natural environment's temperature the established maximum temperature (see Figure 2).
5. After the established timeframe, measure the growth, and sample the colonies to cultivate them at maximum temperature with their respective pH levels (**post-test**).
6. Apply antifungal to all the samples and safely dispose of the products of the experiments.

The proposed procedure (1) uses in-vitro grown colonies to avoid exposing living hosts to fungal infections, (2) applies antifungal agents to every group to safely dispose of the products generated by this experiment, and (3) asks researchers to defined ahead-of-time all the relevant parameters for the experiment and anonymize the samples (blinding) to account for their potential interests and confirmation biases[9].

[8] **#comparisongroups**: The application of this HC sets up different comparison groups for the proposed interventional study: Natural History (for each fungi species) and the Positive Gradient of Temperature groups (for each fungi species and pH level). The proposed groups allow to account for confounding variables and measure practical significance through the pre and post tests.

[9] **#biasmitigation**: The application of this HC clearly states measurements put in place to mitigate possible bias. In particular, measures to account for researcher biases are established: determining all the relevant parameters for the experiment ahead-of-time (even before sampling the fungi species); and once the fungi species are sampled, an anonymization process is executed as a blinding technique.

Expected results and possible interpretations

To enhance the results of the proposed experiment, several controls have been implemented, including generating comparison groups with different base pH levels, a natural evolution group, and fixing the protein/nutrient availability, humidity, and carbon concentration for each group. Future studies should consider variables such as the production of stress-shock proteins and relationships that may arise from predictor variables.

The proposed experiment was inspired by a study that researched antibiotic resistance caused by antibiotic exposure. In that experiment, bacteria achieved significantly higher antibiotic resistance and increased growth rates in environments with high antibiotic concentrations. Similarly, the expected results for the proposed experiment suggest that recurrent and systematic exposure to higher temperatures will lead to a significant increase in thermoresistance as measured by the growth rate of the colony. Furthermore, research has shown that heat is an environmental stressor that accelerates genomic mutations, indicating that fungi may achieve the expected resistance faster than bacteria achieved antibiotic resistance.

Future research can take two directions:

1. Replicate and/or extend the proposed experimental setting to increase both validity and generalizability of the results. This can be achieved by controlling more variables, testing different species of fungi, investigating the relationship between thermoresistance and the production of stress-shock proteins, and antifungal drug tolerance[10].

[10] **#studyreplication**: The application for this HC clearly outlines the steps to replicate the proposed experiment as well as considerations such as nuanced measurements for the dependent variable. The reliability of the results can be strengthened by replicating the experiment with the same sampled fungi species and potentially varying the experimental temperatures and/or accounting for more of the mentioned confounding variables. Similarly, the generalizability of the results can be tested by replicating the experiment with a different sample of fungi species.

2. Investigate the impact of thermoresistant fungi colonies on infection rates. If the predicted increase in thermoresistance is observed, the next step would be to determine whether this translates to an increase in infection rates in warm-blooded organisms. This can be done by exposing the evolved fungi to mammalian cells and measuring the rate of cell infection.[11]

1316 words

[11] **#professionalism**: The application of this HC follows all of the APA conventions and references all of the cited works. Furthermore, it includes all of the works consulted throughout the development and writing of this report.

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