THE BLACK BOX OF PLANT DEMOGRAPHY: SEED GERMINATION AND PATHOGENS

by

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A THESIS

Presented to the Department of Environmental Science
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

December 2019

An Abstract of the Thesis of

Hunter Mackin for the degree of Bachelor of Arts
in the Department of Environmental Science to be taken December 2019

Title: The Black Box of Plant Demography: Seed Germination and Pathogens

Approved: _____

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Most demography studies tend to overlook germination, in favor of seedling survival. However, germination itself is limited by many factors, and remains an essential part of the fitness equation. This study examines seeds of the two native perennial grasses *Festuca roemeri* and *Danthonia californica*, assessing germination rates and seed pathogen richness across both species as well as seed type for *D. californica*, which produces both open-pollinated (chasmogamous) and obligately self-pollinated (cleistogamous) seeds. Germination was found to be significantly lower for cleistogamous seeds, possibly a consequence of a decrease in outcrossing. Germination varied considerably between populations, and we asked whether the environment the seeds developed in, that of the seed mother, influenced these rates. The maximum spring temperature and total spring precipitation each significantly predicted for decreases in germination as they increased, indicating the importance of the growth environment through maternal effects. Further, both soil nitrogen and plant density significantly increased germination rates, likely due to increases in nutrient provision to developing seeds, increased pollen deposition and outcrossing. Pathogen species richness was also assessed for every seed. There were substantial decreases in attack rates for cleistogamous seeds, a plausible benefit of being housed and insulated within their mother's stalks. Pathogen richness depended on the region where the seeds had been collected, and was apparently limited by increases in spring precipitation, a counterintuitive finding that may have been the result of correlation with a variable left unmeasured. There were more pathogens per seed when plant densities were higher.

This is a negative consequence of larger population size, as with these increases in pathogen species richness came decreased germination. These findings suggest that maternal environment plays a large role both in germination and pathogen attack rates, which has implications for present and future climate change. Further, cleistogamous seeds had lower germination rates, but decreased rates of pathogen attack rates, which may serve to counterbalance each other, as the increases in germination for higher populations of higher density may be kept in check by pathogens.

Acknowledgements

I owe an incredible debt of gratitude to Dr. Bitty Roy, without whom this project never would have begun. The advice, guidance, and support given continues to be invaluable. I would also like to thank Tiffany Thornton, who was my partner on this project for all the lab work, and the primary reason the breadth and timeliness of data collection and fungal identification was as extensive and punctual as it was. Zoe Wender also deserves many thanks for all of her help in setting up petri dishes for *D. californica* seeds. Further thanks to Allison Ludden, whose help with culturing saved much time and many headaches. Dr. Ashley Shaw's recommendations for data visualization and analysis were also extremely helpful, and many of the graphs would not have ended up as complete as they were without Graham Bailes, whose research itself also proved invaluable. Additional thanks are due to my secondary advisor Dr. Lauren Hallett, whose expertise on analysis and data visualization was indispensable. Dr. Jeffrey Stone, Dr. George Carroll, and Dr. Roo Vanderbilt's knowledge of the mycological world, and helpfulness with experimentation was both much needed and much appreciated. I would also like to thank my Clark Honors College representative Daphne Gallagher for her willingness to support and dedicate time to this project. This project was made possible by demography sites found by Dr. Bitty Roy, (and a few sites added by Holden Jones), as well as seeds/site data collected by Holden Jones, Bill Morris, Graham Bailes, and Aaron Nelson. I cannot thank you all enough.

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Introduction

The Willamette Valley was historically known for its prairie habitat. Spanning over 1,353,795 ha, the Willamette Valley is nearly 201 km long and around 32-81 km wide (Christy and Alverson 2011). Prior to colonization, prairies were the largest portion of this area, covering 424,606 ha, or 31.4% (Christy and Alverson 2011). Yet today, these prairies cover less than 1% of their historic range within the Willamette Valley (Vesely and Rosenberg 2010). The pressure to conserve these areas is increasing with continued development and the effects of climate change (Helzer 2009).

Two of the most important and widespread native species of grass in these habitats are the perennials *Festuca roemeri* and *Danthonia californica* (Christy and Alverson 2011). *F. roemeri* is a common species to Willamette Valley prairies, and also ranges south to California and north to British Columbia (Wilson et al. 2008). A drought-tolerant species, *F. roemeri* is valuable in restoration projects and for erosion control (Wilson et al. 2008). *D. californica* also has a range from California to British Columbia, though it also extends further eastward through the Rocky Mountains than *F. roemeri* does (Darris and Gonzalves 2019). In addition to being notoriously drought-tolerant and useful in restoration projects and erosion control as well, *D. californica* can tolerate a wide variety of soil types (Darris and Gonzalves 2019). Both species typically dominate prairies together from the Willamette Valley through the Puget Trough (NatureServe 2019).

Much research has been conducted on these species recently, particularly on their response to climate change (Pfeifer-Meister et al. 2013, Pfeifer-Meister et al. 2016, Bailes 2017, Avis et al. 2019). Yet there is yet little known about the germination rates

of these species in natural populations, and the various factors that determine it. One of the primary motivations for this study was this exact reason. Demographic studies often ignore germination, instead focusing on realized fitness, also known as seedling survival (Silvertown et al. 1993, Morris and Doak 1998). To fill this gap, this study was designed, using seeds from an ongoing demographic study of multiple natural populations of both species. There is considerable variation among these natural populations across a climate gradient in both seed production and the resultant seedling numbers (Roy et al. unpublished data).

We first asked if there were differences between the species. We expected that *Danthonia* would have lower germination due to having variable dormancy, in which seeds may need to break multiple dormancy periods through weathering (typically via winter temperatures) as opposed to one dormancy period in *Festuca* and most other genera of grasses (Darris and Gonzalves 2019). We then asked if there were differences between seed type, as *Danthonia* has another particularly unique characteristic of producing two kinds of seeds: self-pollinated seeds requiring only one parent, known as cleistogamous seeds, and typical cross-pollinated seeds with the opportunity to have two individual and separate parents, known as chasmogamous seeds. Cleistogamous seeds were hypothesized have lower germination than their chasmogamous counterparts, as they are both structurally more delicate (Laude 1949) and potentially genetically less robust, due to self-fertilization and inbreeding (Baskin and Baskin 2019).

The germination testing in this study was done indoors in a “common garden” under constant environmental conditions, but we expected that there would be a legacy

of the mother's environment of growth (=maternal effects) on the germinating seeds because the environment the plants grow in determines how well the mothers can provision their seeds (Baskin and Baskin 2019). Environmental conditions where the mother grew that are known to affect germination of their seeds include: temperature and nutrients (Huang et al. 2018), light (Galloway 2001), competition and soil moisture (Germain et al. 2019). For this study, after confirming that there were differences in germination among populations and regions, we used measured differences in plant density, soil nutrients, elevation, and climate of their mothers' respective environments to explain these differences.

Another hypothesis is that pathogens are killing the seeds, not climate, or perhaps in conjunction with climate. In this study, we determine whether these host species and populations vary in terms of both germination and seed pathogen attack rates, what the pathogen species are that are killing the seeds, and how climate affects pathogen interactions. Identifying both the pathogens and their effects on these grass species' survival and reproduction provides an important component within the larger picture of demography and climate change. Populations undergoing warmer temperatures, such as the southernmost populations, could be more physiologically stressed and potentially be more susceptible to infections, for example.

Accounting for what influences the rate of these infections is also an important part of the puzzle. While much is now known about the endophytic communities of each of these species (Bailes 2017), little is known about the effects of pathogen growth on the seeds. We hypothesized that variable seed dormancy might protect *D. californica* seeds better than those of *F. roemeri* via better insulation. Further, cleistogamous seeds

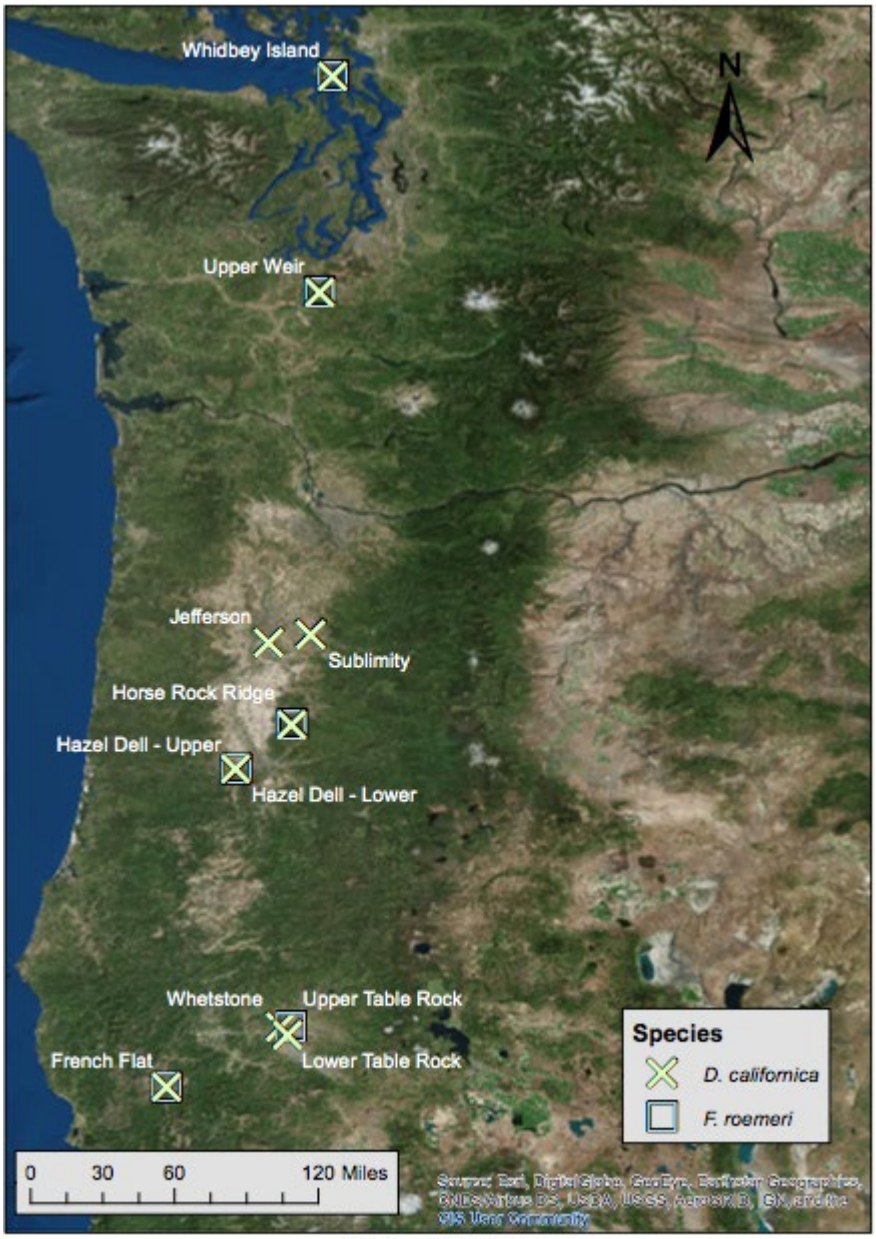
in particular, which are housed within the stalks of the mother, could be even more insulated from pathogens than their chasmogamous counterparts. This hypothesis reflects the presumption that, due to exposure to the elements, chasmogamous seeds are exposed to more air and water borne pathogens than the enclosed cleistogamous seeds, despite the potential for vertical transmission (from mother to seed) of pathogens to cleistogamous seeds (Kover and Clay 1998). In addition, the rates of pathogen attack, as well as the diversity of pathogens, was expected to vary by population and region and thus that environmental variables affecting the seeds and their pathogens prior to seed collection could help explain these differences. We also expected there to be differences in pathogen richness and fungal composition among regions because of significant barriers to dispersal (a mountain range with no suitable prairie habitat just south of Roseburg, Oregon, and the Columbia River between Oregon and Washington (Wilson et al. 2008, Bailes 2017).

Methods

Study Areas

The study design is hierarchical: seed families (mothers and their offspring) within populations and populations within regions along a ~800 km long north to south latitudinal gradient (Bailes 2017). Seeds were collected from six populations of *F. roemeri* and nine populations of *D. californica* (Table 1); when they co-occurred, both species were collected from the same sites. All of the populations, with the exception of *D. californica* from the Jefferson, Sublimity and Whidbey populations of *Danthonia*, were part of a demographic study initiated in 2015 to examine fitness across a climatic gradient running from southern Oregon to Northern Washington. For *D. californica*, both cleistogamous and chasmogamous seeds were collected for each sampled plant.

Figure 1. Map of study sites by species.



Map by Hunter Mackin
Sources: HOPS Project, Graham Bailes, Bitty Roy & Holden Jones

Each site was sampled with 1 m wide transects that were long enough to capture 200 individual adult plants (usually about 25 m/transect). The starting and ending latitude and longitude were recorded for each transect. There was some noticeable variation in seed color, ranging from tan to greenish, potentially indicating maturity at

the time of collection. For this reason, greenness of the seeds was noted on a color scale (1-6 for *D. californica* and 1-3 for *F. roemeri*, with lower numbers tanner and higher numbers greener) at the time of collection. Analyses included this green scale, but since it did not add any explanatory power to any of the models it was thus left out. Plant density was not measured for the Whidbey Island, Sublimity and Jefferson populations, and after analysis determined its importance, these sites were left out of analysis until such data could be collected.

Assessment of Germination

The goal was to test germination and fungal attack of 50 seeds/family/species. However, the numbers varied somewhat due to availability. 90mm petri dishes were lined with filter paper and up to 25 seeds per dish were arranged such that as little contact between the seeds occurred as possible. *F. roemeri* seeds filled up to two petri dishes of 25 seeds per parent of its chasmogamous seeds, and *D. californica* seeds filled up to one petri dish of 25 seeds of chasmogamous seeds and one petri dish of 25 cleistogamous seeds. All petri dishes of each species were subsequently watered with distilled water, placed immediately into cold storage at 4°C for four weeks to break dormancy, and watered additionally when necessary. After this, the seeds were kept at room temperature, while germinating seeds were recorded and removed every two days and petri dishes re-watered as needed.

Environmental Data Collection

Experimental data was collected into spreadsheets and then tidied, analyzed, and graphed using R, specifically the packages tidyverse, MASS, and nlme. Proportion of

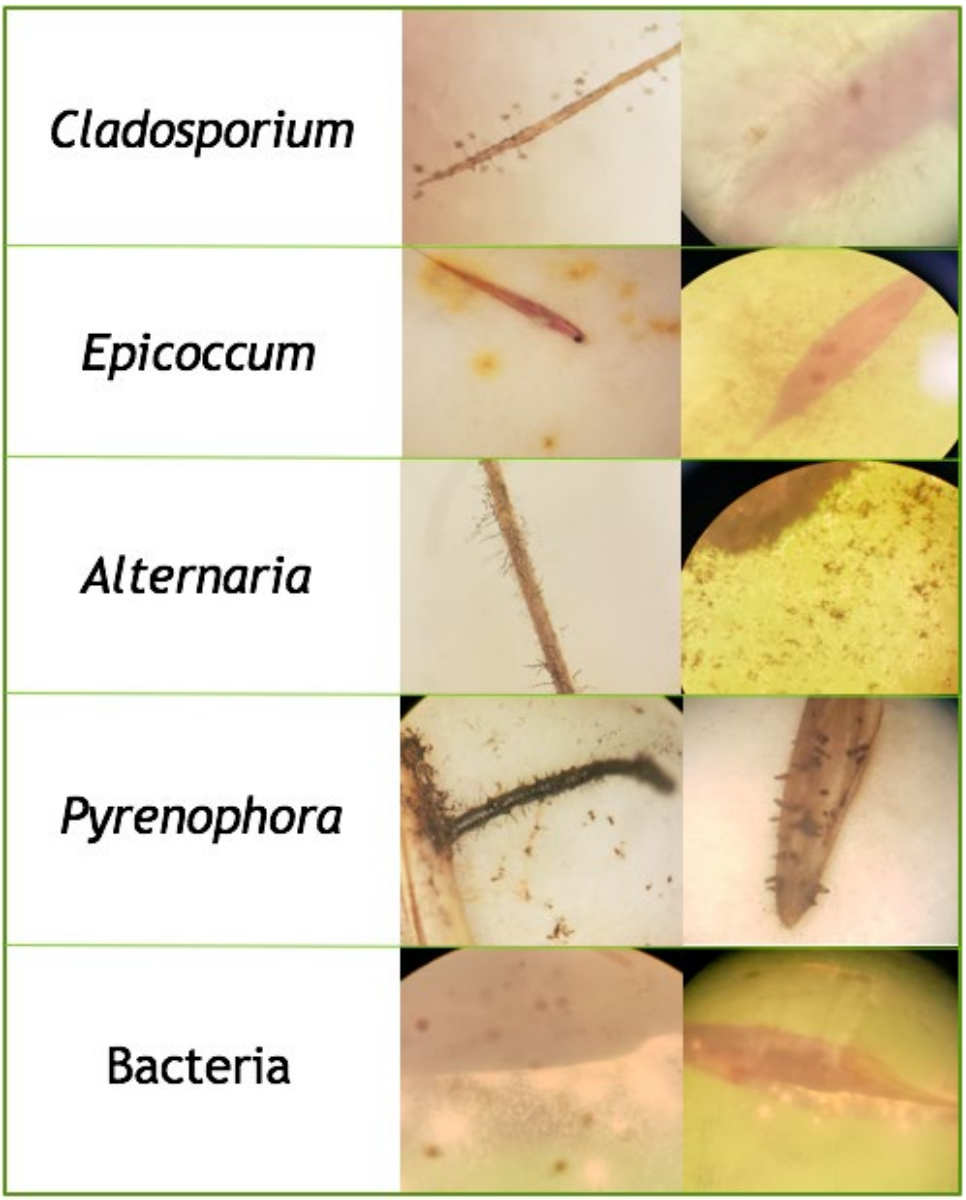
seeds germinated represents the number of seeds with embryos that germinated per parent (and per seed type for *D. californica*). Plant density data was gathered in situ by calculating the counting individuals along a 1m wide transect until 200 plants were identified, forming an index of the number of plants per square meter. Climate data was obtained using the continuously updated Parameter-elevation Regressions on Independent Slopes Model (PRISM) database started in 2004 (PRISM 2018). The climatic variable (and elevation) data were gathered for the first transect of every site, as they did not vary significantly between transects, if they varied at all (the transects were never more than 20 meters apart). Due to the sheer number of climatic variables, only the variables that ended up in the final models are included in Table 2. See the analysis section for how the variables to be used were determined. Data from November 2017 through February 2018 was classified as winter, and data from March 2018 through June 2018 was classified as spring. Specifically, elevation (m), winter and spring precipitation (mm), spring minimum, mean, and maximum temperatures (°C), as well as the winter minimum, mean, and maximum temperatures (°C) were used.

Assessing Pathogen Richness

When the number of germinating seeds approached zero (approximately 6 weeks after removal from 4°C) the fungi on the remaining seeds were identified to a genus or "unknown" using morphology (Fig. 2). Each seed was examined using a dissecting microscope and the presence of every visually identifiable pathogen was noted. At the end of the experiment, the number of kinds of pathogen present were summed for each seed and mother. The averages were then taken for each mother/seed

type, forming an index hereafter referred to as average pathogen species richness or pathogen attack rate.

Figure 2. Photographs of the five major pathogen groups.



Pathogen Identification

To verify identifications and to potentially narrow the identifications to species, we cultured fungi from up to three seeds from each parent plant (for *D. californica*,

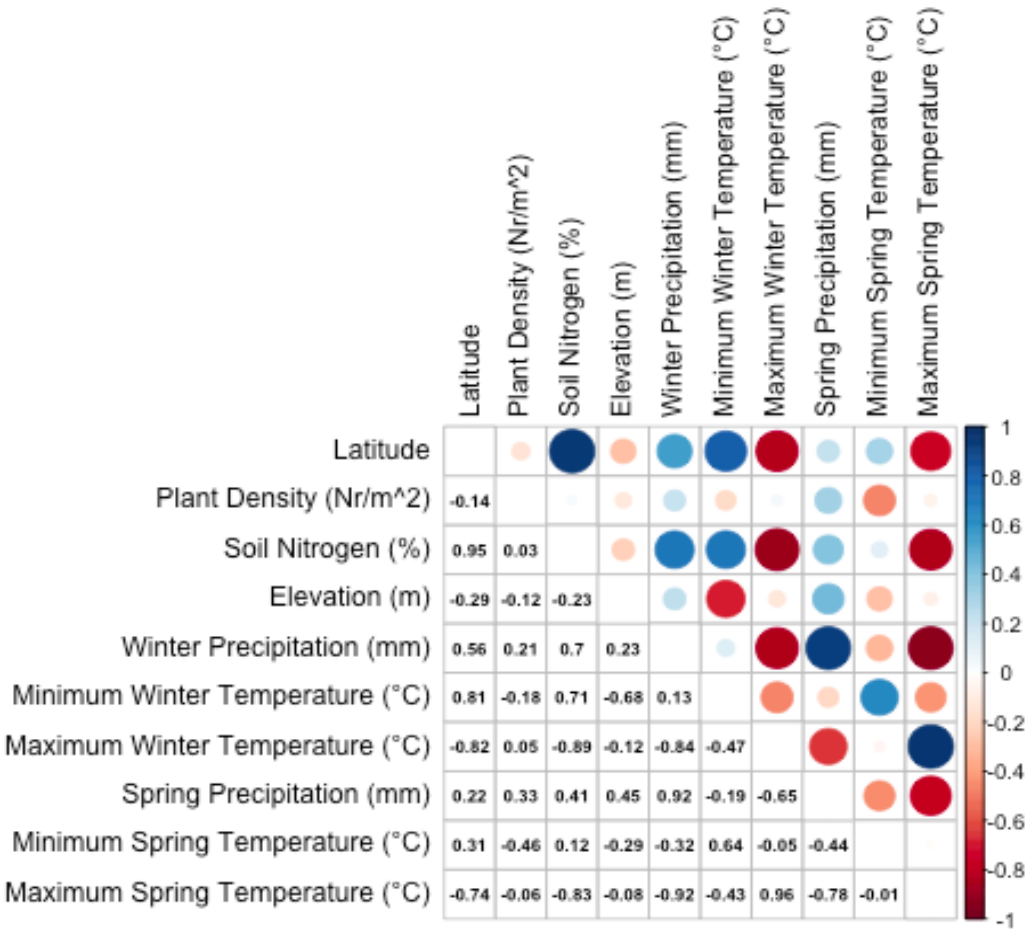
three for cleistogamous and three for chasmogamous seeds from each mother plant). This transfer from the 90mm petri dishes lined with filter paper to 90mm petri dishes filled with solid 2% water agar treated with a mix of antibiotics (20 mL/L of antibiotic solution to water agar, with the antibiotic solution being comprised of 5 g/L of penicillin, 5 g/L of streptomycin, and 1.5 g/L of chloramphenicol) was performed to prevent bacterial contamination, while still promoting growth (Stone et al. 2004). After fungal growth occurred, samples were isolated into 2% malt agar (Stone et al. 2004). After sufficient growth, agar was sampled from near each seed and transferred to 30mm solid 2% malt agar petri dishes to better isolate fungal species while promoting faster growth. As this growth progressed, we morphotyped the cultures (put similar ones together) to reduce the number of cultures we sequenced. After sufficient growth occurred yet again, agar was again sampled and then relocated to 25 mm 2% liquid malt media Erlenmeyer flasks to further isolate species and provide adequate material for extraction. DNA extraction protocol and Sanger sequencing, a technique that identifies only the predominant DNA species present (Serre 2006) was modeled after (Thomas et al. 2016). The overarching laboratory protocol was modeled after (Halbritter et al. 2012). The sequences of the cultured pathogens were cleaned up and aligned using the bioinformatics software Geneious.

Analysis

There were many potential explanatory environmental variables at the sites that could have affected seed provisioning by the plants and pathogen accumulation on the seeds (Fig. 2). To reduce the number of variables used in analysis, we used correlation matrices (see Figure 2) to determine which factors were correlated at a level above

r=0.7; when factors were this strongly correlated the factor that was not specifically in an *a priori* hypothesis was dropped from further consideration.

Figure 3. Correlation matrix of environmental variables.



Displayed using the R package corrplot. Larger circles indicate a higher absolute r-value (correlation coefficient, represented numerically by the bottom left half of numbers). Darker red colors indicate stronger negative correlation, while darker blue colors indicate stronger positive correlation.

Following the criteria of (Quinn and Keough 2010) to control multicollinearity, only VIFs (variance inflation factors) less than 10 were accepted for use in statistical models. VIF analysis (see R Markdown Code for details) concluded that minimum

Winter temperature (°C) and Maximum Spring temperature (°C) could not be in the same model, thus separate models were constructed for them, as well as for soil nitrogen (%), which was evidently correlated as per the correlation matrix (Figure 3).

Generalized linear mixed effects models (GLMMs) were then populated with either the proportion of seeds germinated or the average pathogen species richness as the dependent variables, and species, seed type, spring precipitation (mm), elevation (m), and one from either spring maximum temperature (°C), winter minimum temperature (°C), or soil nitrogen (%) as fixed effects. To determine whether average pathogen species richness affected seed germination, average pathogen species richness was also incorporated as a fixed effect into the models with proportion of seeds germinated as a response.

Results

Germination

Q. 1. Do the grass species differ in germination?

When a simple regression of species on the proportion of seeds germinated was run, we found that *F. roemeri* had an approximately 8.2% higher germination rate than *D. californica* ($P<0.001$). Yet, when the environmental variables that could affect maternal provisioning were included in the statistical models, species appeared to have no significant effect on germination (see Tables 4, 5, and 6).

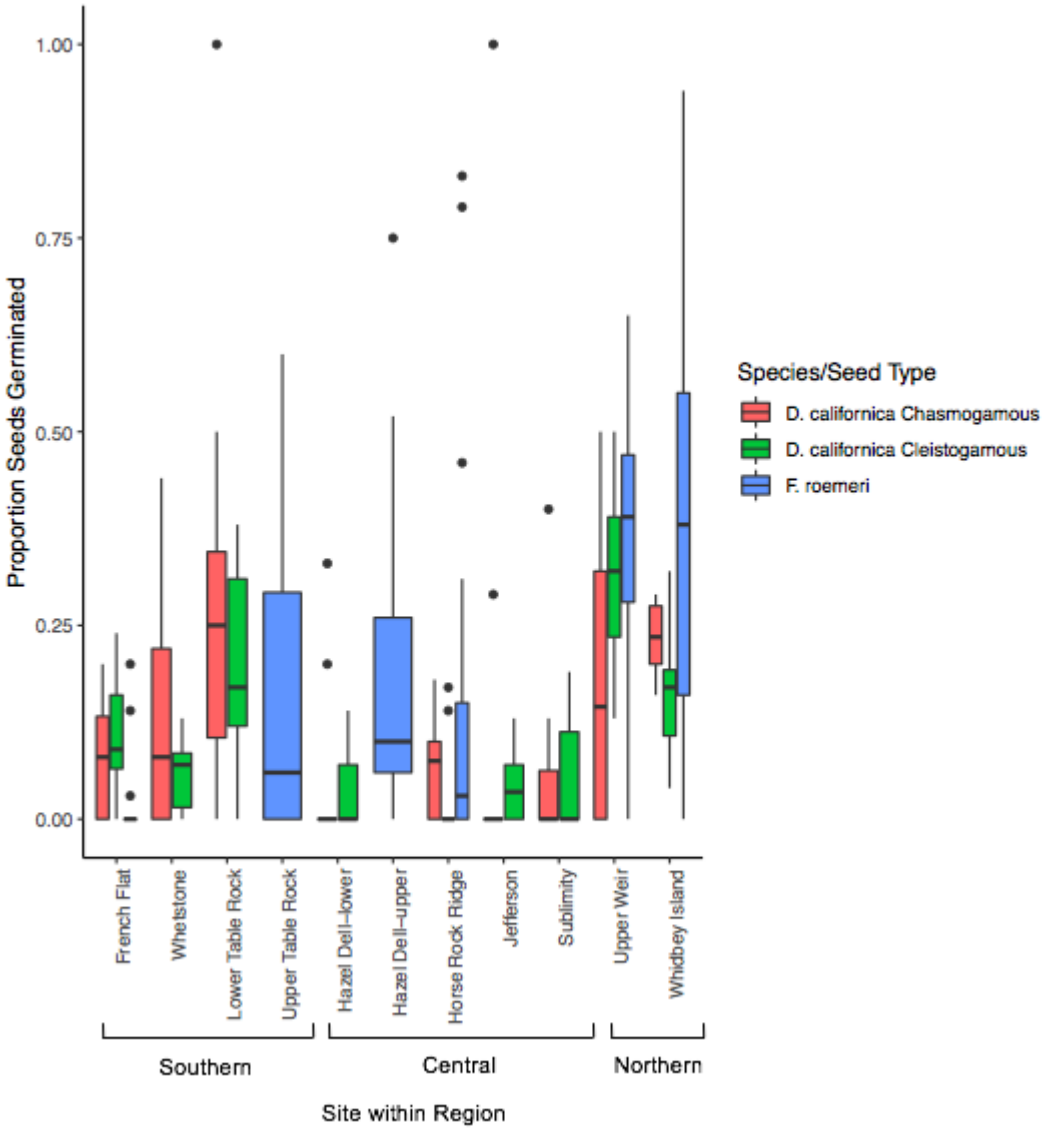
Q. 2. Did the type of seed (enclosed cleistogamous vs. exposed chasmogamous) affect germination rates in Danthonia?

After restricting analysis to solely *D. californica* seeds, simple regression found no significant effect of seed type on germination (estimating a 3.2% decrease with $P = 0.143$). However, when the environmental variables were then included in the models, these findings became marginally significant, estimating a 4.8% to 4.9% decrease in germination for cleistogamous seeds ($P=0.083$ (Table 5) and $P=0.081$ (Table 4) respectively).

Q. 3. Does seed germination depend on maternal family, or on the population from which the seeds came?

Yes; germination depended on both mother and population.

Figure 4. Boxplots of proportion of seeds germinated as a function of site.



Sites are ordered by latitude, increasing from left to right, and further sectioned by region. The black dots indicate mother-seed families with germination rates considered outliers.

Visual representation suggested massive differences between and within sites across a latitudinal gradient (Figure 4). This effect appeared to be U-shaped across gradients for *D. californica* chasmogamous and cleistogamous seeds, and linearly increasing for *F. roemeri* (Figure 4). Incorporating the random effects of mother nested

within site (population) vastly improved the R^2 (marginal to conditional) in every single model (see Tables 3, 4, and 5). However, these effects seen in Fig. 4 were much better explained by environmental variables, see below.

Q. 4. If there are differences in germination among populations, can they be explained by environmental variables such as climate, elevation, soil nutrients, and plant density?

Following incorporation into GLMMs, total spring precipitation was found to be a significant reducer of germination in the models including maximum spring temperature and soil nitrogen (though not in the minimum winter temperature model), estimating a drop in germination of 0.23 % to 0.37% for millimeter increase in total spring precipitation (P=0.042 (Table 5) and P=0.014 (Table 3) respectively). Spring maximum temperature was found to be significant as well, with an estimated decrease of 0.39% decrease in germination for every increase of maximum spring temperature of 1 °C (P=0.029 (Table 3)). Soil nitrogen, on the other hand, estimates a 28.6% increase in germination for every rise in soil nitrogen of 1% (P=0.049 (Table 5)). Plant density proved a significant factor in every model it was included in, estimating a 0.32% to 0.39% increase in germination for every increase in the number of plants per square meter by 1 (P=0.008 (Table 5), P=0.004 (Table 4), P=0.003 (Table 3)).

Seed Pathogens

Q. 5. Do the grass species differ in seed pathogen richness?

After running a simple regression of species on average pathogen species richness, we found that *F. roemeri* seeds are predicted to have approximately 0.3627 more pathogens of seeds ($P<0.001$). Yet after including environmental variables (and cleistogamy), the models ran predicted either slightly positive or slightly negative effects, though none of these results were significant (see Tables 3, 4, and 5).

Q. 6. Did the type of seed (enclosed cleistogamous vs. exposed chasmogamous) affect seed pathogen richness in Danthonia?

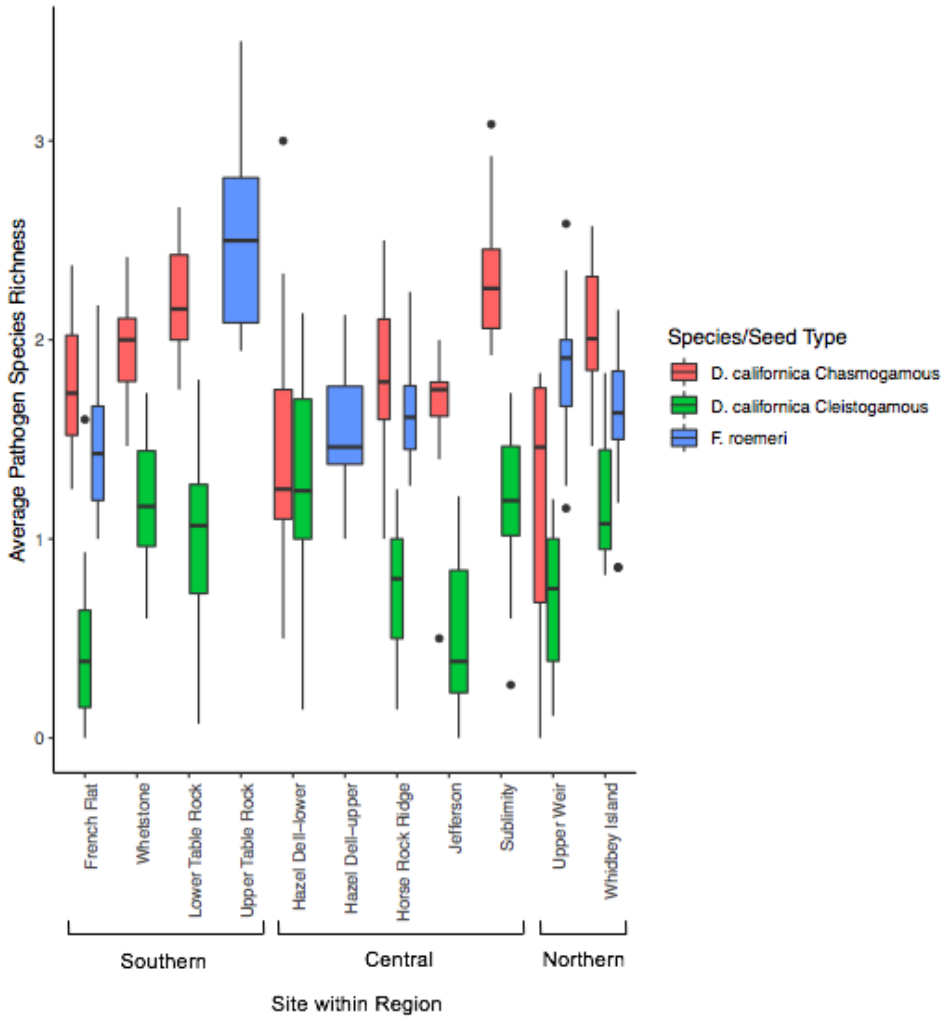
Yes; cleistogamy was the most significant predictor of average pathogen species richness, as both simple regression and GLMM analysis found. When subsetting the larger data set to strictly *D. californica* seeds, a simple regression of seed type on average pathogen species richness estimated for 0.8710 less pathogens per seed ($P<0.001$). GLMMs congruently estimated decreases in the average number of pathogens per seed from 0.8768 to 0.8769 ($P<0.001$ (Table 4), $P<0.001$ (Table 3) respectively).

Q. 7. Does seed pathogen richness depend on maternal family, or on the population from which the seeds came?

Yes; when looking across sites ordered by latitude, it becomes apparent that there's considerable variation of average pathogen species richness within regions (Figure 5), suggesting that there may be many factors at play, or perhaps those less related to latitude (such as plant density). The incorporation of seed mother nested

within site thereby improved the R^2 (from marginal to conditional) for every single model ran (see Tables 3, 4, and 5).

Figure 5. Boxplots of average pathogen species richness as a function of site



Sites are ordered by latitude, increasing from left to right, and further sectioned by region. The black dots indicate mother-seed families with average pathogen species richness values considered outliers.

Q. 8. If there are differences in germination among populations, can they be explained by environmental variables such as climate, elevation, soil nutrients, and plant density?

GLMMs analogous to those run with germination rate (primarily with predictor variables switched, though a model with soil nitrogen was excluded for lack of

justification), and Spring precipitation were thereby found to predict significant decreases in average pathogen species richness anywhere from 0.0090 to 0.0101 pathogen species per seed, per millimeter increase in absolute precipitation (P=0.012 (Table 4), P=0.033 (Table 3) respectively). A marginally significant relationship to elevation was also present, with an estimated increase in average pathogen species richness of 0.0016 to 0.0018 (P=0.085 (Table 3), P=0.051 (Table 4) respectively) per meter rise in elevation. Plant density estimated for increases in average pathogen species richness of 0.0080 to 0.0082 (P=0.001 (Table 3), P=0.009 (Table 4) respectively).

Q. 9. Is high pathogen richness associated with poorer germination of either grass species?

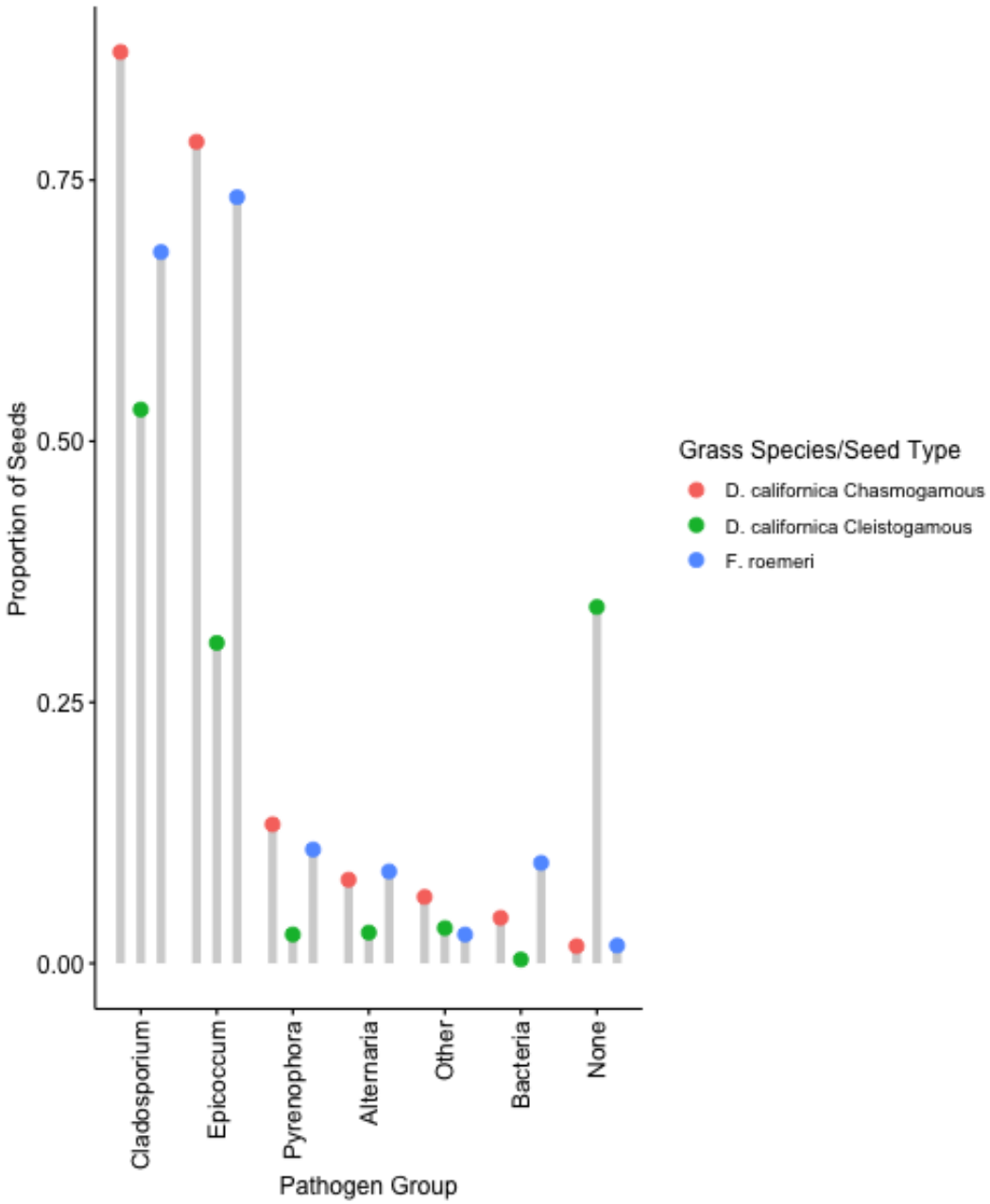
Yes; average pathogen species richness was a significant predictor of germination rates for every model, except when soil nitrogen was included (which still showed a marginally significant trend in line with the others (P=0.090 (Table 5)). Estimated decreases in germination rate ranged from 4.55% to 4.60% per increase in the average pathogen species richness by 1 (P=0.033 (Table 4), P=0.030 (Table 3) respectively).

Q. 10. What are the fungal pathogens on the seeds?

The primary pathogens identified morphologically were those of the genera *Cladosporium*, *Epicoccum*, *Pyrenophora*, *Alternaria* (see Table 6 and Figure 6). These identifications are in the process of being verified (or not) by Sanger sequencing, as well as many of the other fungal species left unidentified in the "Other" category.

Cleistogamous seeds can be seen to have lower proportions of seeds identified for each pathogen type (apart from "Other" in *F. roemeri*), though are by far the leading proportion in the "None" category (Figure 6).

Figure 6. Proportion of grass species/seed type with observations of each pathogen group.



Color of dots represent each grass species/seed type.

Discussion

This experiment provided evidence that the environment of maternal growth in plants affects seed germination and pathogen attack, in ways that were both expected and unexpected. Firstly, counter to predictions, on average, germination rates of *F. roemeri* seeds only significantly differed from *D. californica* seeds prior to the incorporation of environmental variables. This suggests that, while there may be some minimal effect of variable dormancy in *Danthonia* that this experiment was not able to account for, these two species may be deemed roughly equivalent in terms of the probability of germination.

Secondly, as predicted, cleistogamous seeds had reduced germination rates relative to chasmogamous seeds in *Danthonia*. Potential reasons why include the genetic consequences of having only one parent (Baskin and Baskin 2019), seed coats being less tolerant to harsher conditions (Laude 1949), or even from a combination of both effects. This is the second species of *Danthonia* to be shown to have lower germination of cleistogamous seeds, the other being *Danthonia spicata* (Clay 1983). However, the genus *Danthonia* is an exception to a broader trend of cleistogamous seeds germinating better than chasmogamous seeds (Clay 1983, Baskin and Baskin 2019). It would be interesting to follow the fates of plants produced by the two different seed types in this genus, as otherwise it is difficult to understand why this particular mixed mating system persists. It should be noted that there was no apparent evidence that many of the cleistogamous seeds were non-viable apart from the lack of germination; therefore, a future study ought to use tetrazolium, which turns living cells red, to test whether seeds are still viable (Quinn and Keough 2010). We did attempt to

incorporate tetrazolium into this study, and no living cells were found to be present probably due to stopping watering a week prior to this attempt; we likely killed the seeds.

Germination rates varied dramatically (Figures 4 and 5) with region, population, and mother all explaining some variation. By then including specific environmental variables in the statistical models, we were able to determine what aspects of the environment were contributing most to germination. Maximum spring temperature was one of the puzzle pieces, predicting for decreases in germination for every uptick in degree in the mother's environment. The work of (Roberts 1988) informs this effect; seeds typically germinate better under warmer conditions up to an optimal temperature, and then decrease in germination as they approach a ceiling temperature (where no seeds germinate). Our data therefore suggest that maximum spring temperatures are in-between that optimal temperature and ceiling temperature, and that increasing Spring temperatures pushes it closer to the ceiling.

Curiously, higher spring precipitation predicted for reduced germination, which pushes back on what is currently known about the effect of precipitation on perennial bunchgrasses. Sufficient precipitation can push germination rates up to 100% in some instances (Boyd and Lemos 2015). However, the study conducted by (Boyd and Lemos 2015) looked at the effects of such environmental variables on the mother, rather than the seeds themselves. One study looked into the maternal effects of precipitation (and nitrogen deposition) on the perennial forb *Potentilla tanacetifolia* found that seed production increased, while seed mass and germination rate remained the same (Li et al. 2011). Another study, which focused on the perennial grass *Stipa krylovii*, observed

decreased seed viability and seed mass, and no changes in seed production, with higher levels of precipitation (Ronnenberg et al. 2011), though the mechanism was left undiscovered. Therefore, seed viability may have been reduced with increased precipitation, a phenomenon worth further study.

While there has been research on how increases soil nitrogen improves the germination of grasses, allowing them to outcompete forbs and lower species diversity (Bird and Choi 2017), such studies typically focus on how nitrogen affects seeds directly grown in soil (Christensen et al. 2015). Less research is currently available on how soil nutrients affect a mother's ability to produce viable seeds, though one study, focusing on the nitrophilous weed *Rumex obtusifolius*, found that nitrogen addition to the soil in which a seed mother grew vastly improved laboratory their seeds' germination rates (Hrdlickova et al. 2011). Our study corroborates those findings, as seeds collected from sites with higher soil nitrogen had considerably higher germination rates.

Plant density in the mother's population was the most significant estimator of germination, predicting increases in germination for every additional plant per square meter. One of the likely reasons for this is pollen limitation. Grasses are wind pollinated, so this effect is not due to a reduction in pollinators, but wind-pollinated plants have a threshold population size below which pollination is less and less likely (Knight et al. 2005). Furthermore, small populations are likely to be composed of closely related individuals, also facilitating inbreeding and its ill effects on genetics. The greater gene flow from outcrossing allows populations to avoid the persistence of detrimental alleles (Wright 1948), prevent genetic bottlenecks (Bryant et al. 1986), and

develop mechanisms for resistance to various pathogens at higher rates (Marden et al. 2017). However, our study also demonstrated how plant density can work to the benefit of pathogens.

Before diving deeper into that finding, first it should be noted that pathogen attack rates only varied between species before seed type and environmental variables were controlled for, indicating that chasmogamous *D. californica* seeds had no particular advantage over *F. roemeri* seeds, regardless of dormancy traits. Cleistogamy, on the other hand, was by far the largest predictor of pathogen attack rates, reducing the average number of pathogen species per seed by nearly one. While the mechanism for this is yet unconfirmed, the insulation from being housed within the mother's stalks is posited to be the most likely reason.

Attack rates varied between populations as well, and are influenced by several environmental variables, some in shockingly similar ways to germination. Notably, higher spring precipitation predicted for decreased numbers of pathogens per seed. This is contrary to a great body of literature, which finds improved fungal growth during warm and wet conditions across the board (Hawkes et al. 2011, Landesman and Dighton 2011, Yamashita et al. 2012, Prihatini et al. 2015). One study even found an increased presence of *Bipolaris* (a genus now known as *Pyrenophora* – which our study found a large amount of) in areas of higher precipitation (Lacicowa and Pieta 2013). The precipitation being discussed here was that of the mother's environment, which is what the seed experienced when it was formed; we quantified the number of pathogens under a constant environment. The underlying cause of this relationship remains unknown regardless, but it is worth further exploration. Areas of higher elevation, on

the other hand, had higher pathogen attack rates relative to areas of lower elevation. This is congruent with (Myster 2018), which found decreased herbivory but increased rates of pathogen attack at higher elevations, possibly due to greater wind dispersal.

Plant density heavily influenced the number of pathogen species observed, larger numbers of kinds of pathogens occurring in larger populations. While some outbreaks can heavily infect smaller populations, higher plant density allows pathogens to spread more quickly between hosts, and they are more likely to stumble on larger populations to begin with (Burdon 1987). This increase in plant density may very well decrease the positive effect of germination that higher plant density demonstrates, as these pathogen attack rates were found to significantly reduce the rates of germination in these populations. Considering that as the average pathogen species richness of seeds of a given mother increased, the proportion of seeds germinated decreased, this lowered richness in cleistogamous seeds could provide a buffer against this effect on germination. However, further experimentation would need to be done to verify that this effect of pathogens on germination is causal; no controls (germinated seeds) were kept and examined for fungal richness, as fungal richness was catalogued in a relatively short time period for consistency's sake. Additionally, fungal cultures from the seeds remain, therefore re-inoculation of these pathogens onto new seeds could provide insights into each species of pathogen's effects on germination, following rigorous sterilization of new seeds. Were this experiment to take place, multiple species could also be introduced for a single seed to test the idea more explicitly that more kinds of pathogens decrease fitness, as has been found in other studies (Roy et al. 2011). Invasive species, alternatively, have been shown to bring more pathogens to the table, and weather them

better (Wilson et al. 2014). Time is of the essence to better understand these effects; with additional pathogens and increasing climate change, having more informed restoration strategies may well be able to mitigate them.

Supplements

Tables

Table 1. Location of the study sites and transects.

Site	Region	Species	Transect	GPS Start Lat	GPS Start Long	GPS End Lat	GPS End Long
French Flat	Southern	*F. roemeri*	1	N 42.10061	W 123.63470	N 42.10077	W 123.63477
French Flat	Southern	*D. californica*	2	N 42.10063	W 123.63541	N 42.10059	W 123.63557
French Flat	Southern	*D. californica*	1	N 42.10083	W 123.63493	N 42.10090	W 123.63479
Whetstone	Southern	*D. californica*	3	N 42.41957	W 122.90706	N 42.41939	W 122.90707
Whetstone	Southern	*D. californica*	2	N 42.41961	W 122.90685	N 42.41938	W 122.90685
Whetstone	Southern	*D. californica*	1	N 42.42040	W 122.90722	N 42.42036	W 122.90724
Lower Table Rock	Southern	*D. californica*	1	N 42.46811	W 122.94635	N 42.46787	W 122.94623
Upper Table Rock	Southern	*F. roemeri*	1	N 42.46857	W 122.88324	N 42.46873	W 122.88322
Hazel Dell b (lower)	Central	*D. californica*	1	N 44.01979	W 123.21823	N 44.01955	W 123.21808
Hazel Dell a (lower)	Central	*F. roemeri*	1	N 44.02518	W 123.21579	N 44.02493	W 123.21581
Horse Rock Ridge	Central	*D. californica*	1	N 44.29804	W 122.87776	N 44.29807	W 122.87777
Horse Rock Ridge	Central	*F. roemeri*	1	N 44.29871	W 122.87784	N 44.29874	W 122.87820
Horse Rock Ridge	Central	*D. californica*	2	N 44.29877	W 122.87984	N 44.2988	W 122.87996
Jefferson	Central	*D. californica*	1	N 44.787292	W 123.018684	N 44.787167	W 123.017999
Sublimity	Central	*D. californica*	1	N 44.841218	W 122.767179	N 44.841263	W 122.76488
Upper Weir (JBLM)	Northern	*D. californica*	1	N 46.90908	W 122.7114	N 46.90909	W 122.71178
Upper Weir (JBLM)	Northern	*D. californica*	2	N 46.90931	W 122.70974	N 46.90932	W 122.70932
Upper Weir (JBLM)	Northern	*F. roemeri*	1	N 46.90952	W 122.70979	N 46.90978	W 122.70995
Upper Weir (JBLM)	Northern	*D. californica*	3	N 46.90956	W 122.7082	N 46.9096	W 122.70792
Whidbey Island	Northern	*F. roemeri*	2	N 48.20977	W 122.62394	N 48.20992	W 122.62411
Whidbey Island	Northern	*F. roemeri*	1	N 48.20979	W 122.62335	N 48.20977	W 122.62348
Whidbey Island	Northern	*D. californica*	1	N 48.210373	W 122.629092	N 48.210331	W 122.627038

Sites are ordered by increasing starting latitude. Central=the Willamette Valley of Oregon, which is central relative to the other two regions, Southern=Siskiyou region of southern Oregon, and Northern=Puget Trough of Washington, north of the Columbia River. 1. Latitude and longitude from Bailes (2017) and Holden (unpub. data).

Table 2. Environmental data for each site, ordered by increasing starting latitude.

Site Name	Species	Region	Elevation (m)	Winter Minimum Temperature (°C)	Spring Maximum Temperature (°C)	Spring Precipitation (mm)	Soil Nitrogen (%)	Plant Density (Nr/m^2)
French Flat	*F. roemeri*	Southern	464	-2.5	26.6	110.935	0.15	18.76
French Flat	*D. californica*	Southern	364	-2.5	26.6	110.935	0.15	42.82
Whetstone	*D. californica*	Southern	377	-1.2	27.6	32.5375	0.12	0.53
Lower Table Rock	*D. californica*	Southern	478	-1.5	27	35.65	0.19	6.01
Upper Table Rock	*F. roemeri*	Southern	390	-1.2	27.6	33.71	0.2	13.32
Hazel Dell b (lower)	*D. californica*	Central	157	-0.4	23.4	61.975	0.3	16.8
Hazel Dell a (upper)	*F. roemeri*	Central	156	-0.7	23.3	65.3	0.3	9.33
Horse Rock Ridge	*D. californica*	Central	570	-1.2	21.3	131.8825	0.5	1.47
Horse Rock Ridge	*F. roemeri*	Central	570	-1.2	21.3	131.8825	0.5	11.28
Jefferson	*D. californica*	Central	202	-0.2	23	77.2575	-	-
Sublimity	*D. californica*	Central	214	-0.3	22.7	85.625	-	-
Upper Weir (JBLM)	*D. californica*	Northern	152	-0.4	21.5	65.985	1.06	1.51
Upper Weir (JBLM)	*F. roemeri*	Northern	152	-0.4	21.5	65.985	1.06	21.5
Whidbey Island	*D. californica*	Northern	39	1	18.8	48.2725	-	-
Whidbey Island	*F. roemeri*	Northern	39	1	18.8	48.2725	-	23.16

Source: (Bailes 2017, PRISM 2018).

Table 3: GLMM including maximum spring temperature.

	Estimate	df	t-value	p-value
Part A: Response: Proportion of Seeds Germinated per Mother				
(Intercept)	1.238	4.36	4.30	0.011 *
Species: *F. roemeri*	0.0269	117.3	0.88	0.380
Seed Type: Cleistogamous	-0.0485	58.1	-1.78	0.081 .
Spring Precipitation (mm)	-0.0037	5.4	-3.56	0.014 *
Spring Max Temperature (°C)	-0.0388	4.2	-3.24	0.029 *
Plant Density (Nr/m^2)	0.0039	116.4	3.09	0.003 **
Elevation (m)	0.0004	5.3	1.75	0.138
Average Pathogen Richness	-0.0460	116.4	-2.20	0.030 *
Part B: Response: Average Pathogen Richness per Seed per Mother				
(Intercept)	2.271	4.5	2.29	0.076 .
Species: *F. roemeri*	-0.0598	210.8	0.74	0.460
Seed Type: Cleistogamous	-0.8769	270.0	-12.21	0.000 ***
Spring Precipitation (mm)	-0.0101	4.8	-2.95	0.033 *
Spring Max Temperature (°C)	-0.0184	4.5	-0.44	0.677
Plant Density (Nr/m^2)	0.0080	235.6	2.60	0.001 **
Elevation (m)	0.0016	4.8	2.16	0.085 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Part A: Marginal R^2 = 0.261, Conditional R^2 = 0.684

Part B: Marginal R^2 = 0.484, Conditional R^2 = 0.584

Number of Observations = 280

Table 4: GLMMs including minimum winter temperature.

	Estimate	df	t-value	p-value
Panel A: Response: Proportion of Seeds Germinated per Mother				
(Intercept)	0.2768	8.3	3.12	0.014 *
Species: *F. roemeri*	0.0246	176.6	0.77	0.441
Seed Type: Cleistogamous	-0.0483	57.8	-1.76	0.083 .
Spring Precipitation (mm)	-0.0013	5.4	-1.34	0.234
Winter Min Temperature (°C)	0.1029	4.4	1.84	0.133
Plant Density (Nr/m^2)	0.0039	159.5	2.96	0.004 **
Elevation (m)	0.0003	5.0	0.912	0.404
Average Pathogen Richness	-0.0455	114.7	-2.16	0.033 *
Panel B: Response: Average Pathogen Richness per Seed per Mother				
(Intercept)	1.785	5.7	9.33	0.000 ***
Species: *F. roemeri*	0.0596	198.4	0.74	0.460
Seed Type: Cleistogamous	-0.8768	270.1	-12.21	0.000 ***
Spring Precipitation (mm)	-0.0090	4.8	-3.93	0.012 *
Winter Min Temperature (°C)	0.1164	4.4	0.88	0.426
Plant Density (Nr/m^2)	0.0082	230.4	2.65	0.009 **
Elevation (m)	0.0018	4.77	2.59	0.051 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Panel A: Marginal R^2 = 0.198, Conditional R^2 = 0.695

Panel B: Marginal R^2 = 0.508, Conditional R^2 = 0.584

Number of Observations = 280

Table 5: GLMM including soil nitrogen.

	Estimate	df	t-value	p-value
Part A: Response: Proportion of Seeds Germinated per Mother				
(Intercept)	0.1275	5.7	1.53	0.181
Species: *F. roemeri*	0.0234	102.4	0.82	0.416
Seed Type: Cleistogamous	-0.0399	61.1	-1.48	0.144
Spring Precipitation (mm)	-0.0023	4.0	-2.94	0.042 *
Elevation (m)	0.0002	3.9	1.31	0.264
Soil Nitrogen (%)	0.2857	2.9	3.29	0.049 *
Plant Density (Nr/m^2)	0.0032	86.1	2.73	0.008 **
Average Pathogen Richness	-0.0351	125.8	-1.71	0.090 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Part A: Marginal R^2 = 0.263, Conditional R^2 = 0.639

Part B: Marginal R^2 = 0.505, Conditional R^2 = 0.60

Number of Observations = 256

Table 6: Results of seed pathogen morphotyping.

Species/Seed Type	Pathogen Group	Count	Proportion of Seeds Per Species/Seed Type
F. roemeri	*Cladosporium*	2075	0.6812
F. roemeri	*Epicoccum*	2235	0.7337
F. roemeri	*Alternaria*	268	0.0880
F. roemeri	*Pyrenophora*	332	0.1090
F. roemeri	Bacteria	293	0.09619
F. roemeri	Other	84	0.0276
F. roemeri	None	52	0.0171
F. roemeri	Total	3046	1.0000
D. californica Chasmogamous	*Cladosporium*	741	0.8728
D. californica Chasmogamous	*Epicoccum*	668	0.7868
D. californica Chasmogamous	*Alternaria*	68	0.0801
D. californica Chasmogamous	*Pyrenophora*	113	0.1331
D. californica Chasmogamous	Bacteria	37	0.0436
D. californica Chasmogamous	Other	54	0.0636
D. californica Chasmogamous	None	14	0.0165
D. californica Chasmogamous	Total	849	1.0000
D. californica Cleistogamous	*Cladosporium*	864	0.5304
D. californica Cleistogamous	*Epicoccum*	500	0.3069
D. californica Cleistogamous	*Alternaria*	48	0.0295
D. californica Cleistogamous	*Pyrenophora*	45	0.0276
D. californica Cleistogamous	Bacteria	6	0.0037
D. californica Cleistogamous	Other	55	0.0338
D. californica Cleistogamous	None	556	0.3413
D. californica Cleistogamous	Total	1629	1.0000

R Markdown Code

````
---
title: "blackbox"
output: html_document
---

*Starting packages and importing Excel sheets*

```{r}
library(tidyverse)
library(ggpubr)
library(car)
library(corrplot)
library(MuMIn)


blackboxsplit <- read_csv("Black box cleist_chas_fesroe dataset - Sheet1.csv")
blackboxmatrixrenamed <- read_csv("Renamed Black box matrix - Sheet1.csv")
bothspeciespathogenpres <- read_csv("Both Species Pathogen Count -
Sheet1.csv")
```




*Ordering Sites by Latitude on a Gradient from Lower to Higher*

```{r}
````
````

```
blackboxsplit <- blackboxsplit %>%
  mutate(SiteName = factor(SiteName, levels = c("French Flat",  "Whetstone", "Lower Table Rock", "Upper Table Rock", "Hazel Dell-lower", "Hazel Dell-upper", "Horse Rock Ridge", "Jefferson", "Sublimity", "Upper Weir", "Whidbey Island")))
```

Graphs

```{r}
# Proportion of Seeds Germinated Boxplot
propgermboxplots <- ggplot(blackboxsplit, aes(x=SiteName, y=PropGerm)) + geom_boxplot(aes(fill=SpeciesSeedType)) + theme_classic() + labs(x="", y="Proportion Seeds Germinated") + theme(axis.text.x = element_text(angle = 90, hjust = 1)) + guides(fill=guide_legend(title="Species/Seed Type"))
ggsave(filename = "propgermboxsplots.pdf", plot=propgermboxplots)

# Average Pathogen Species Richness Boxplot
pathogenrichboxplots <- ggplot(blackboxsplit, aes(x=SiteName, y=APSR)) + geom_boxplot(aes(fill=SpeciesSeedType)) + theme_classic() + labs(x="", y="Average Pathogen Species Richness") + theme(axis.text.x = element_text(angle = 90, hjust = 1)) +  guides(fill=guide_legend(title="Species/Seed Type"))
ggsave(filename = "pathogenrichboxplots.pdf", plot=pathogenrichboxplots)

# Total Pathogen Count Dot Chart
png(file="pathogencountdotchart.png",width=500,height=600)
```

ggdotchart(bothspeciespathogenpres, x="PathogenMorpho", y="PropPresence", color="GrassSpeciesSeedType", size=3, sorting = "des", sort.by.groups = TRUE, add = "segments", add.params = list(color = "lightgray", size = 2), group = "GrassSpeciesSeedType", position = position_dodge(0.8), legend="right") + labs(x="Pathogen Group", y="Proportion of Seeds") + guides(color=guide_legend(title="Grass Species/Seed Type")) + theme(axis.text.x = element_text(angle = 90, hjust = 1))

```

*Model Selection - Correlation Plot and VIF Analysis*

```{r}
#Initial VIF Analysis
VIFtot_propgerm <- lm(PropGerm~SpeciesName + Latitude + SeedType + SpringPPT + SpringTmaxC + SpringTminC + WinterPPT + WinterTmaxC + WinterTminC + LastyrElevation + SoilPercentN + PlantDensity + APSR, data=blackboxsplit)
alias(VIFtot_propgerm)
vif(VIFtot_propgerm)
VIFtot_APSR <- lm(APSR~SpeciesName + SeedType + Latitude + SpringPPT + SpringTmaxC + SpringTminC + WinterPPT + WinterTmaxC + WinterTminC + LastyrElevation + PlantDensity + SoilPercentN + PropGerm, data=blackboxsplit)
vif(VIFtot_APSR)
#Correlation Plot
blackboxmatrix <- as.matrix(blackboxmatrix)
```
```

```
blackboxmatrixrenamed <- as.matrix(blackboxmatrixrenamed)
blackboxcor <- cor(blackboxmatrixrenamed, use = 'na.or.complete', method = 'spearman') # you can use spearman, kendall, or pearson's)

png("corrplot.png", width = 480,height = 500)
corrplot.mixed(blackboxcor, lower.col = 'black', number.cex = 0.65, tl.pos = 'lt', tl.col = "black")

#Proportion of Seeds Germinated Final VIF analyses
VIF_propgerm_springmaxwintermin <- lm(PropGerm~SpeciesName + SeedType + SpringPPT + SpringTmaxC + WinterTminC + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_propgerm_springmaxwintermin)

VIF_propgerm_springmaxsoiln <- lm(PropGerm~SpeciesName + SeedType + SpringPPT + SpringTmaxC + SoilPercentN + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_propgerm_springmaxsoiln)

VIF_propgerm_springmax <- lm(PropGerm~SpeciesName + SeedType + SpringPPT + SpringTmaxC + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_propgerm_springmax)
```

```
VIF_propgerm_winterminsoiln <- lm(PropGerm~SpeciesName + SeedType +
SpringPPT + WinterTminC + SoilPercentN + PlantDensity + APSR,
data=blackboxsplit)
vif(VIF_propgerm_winterminsoiln)

VIF_propgerm_wintermin <- lm(PropGerm~SpeciesName + SeedType +
SpringPPT + WinterTminC + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_propgerm_wintermin)

VIF_propgerm_soiln <- lm(PropGerm~SpeciesName + SeedType + SpringPPT
+ WinterTminC + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_propgerm_soiln)

#Average Pathogen Species Richness Final VIF Analyses
VIF_richness_springmaxwintermin <- lm(APSR~SpeciesName + SeedType +
SpringPPT + SpringTmaxC + WinterTminC + PlantDensity, data=blackboxsplit)
vif(VIF_richness_springmaxwintermin)

VIF_richness_springmaxsoiln <- lm(APSR~SpeciesName + SeedType +
SpringPPT + SpringTmaxC + SoilPercentN + PlantDensity, data=blackboxsplit)
vif(VIF_richness_springmaxsoiln)
```

```
VIF_richness_springmax <- lm(APSR~SpeciesName + SeedType + SpringPPT
+ SpringTmaxC + PlantDensity, data=blackboxsplit)
vif(VIF_richness_springmax)

VIF_richness_winterminsoiln <- lm(APSR~SpeciesName + SeedType +
SpringPPT + WinterTminC + SoilPercentN + PlantDensity, data=blackboxsplit)
vif(VIF_richness_winterminsoiln)

VIF_richness_wintermin <- lm(APSR~SpeciesName + SeedType + SpringPPT
+ WinterTminC + PlantDensity + APSR, data=blackboxsplit)
vif(VIF_richness_wintermin)

VIF_richness_soiln <- lm(APSR~SpeciesName + SeedType + SpringPPT +
WinterTminC + PlantDensity, data=blackboxsplit)
vif(VIF_richness_soiln)
```

Simple Regressions and Creating D. californica Data Frame Subset

```{r}
#Species Regression for Proportion of Seeds Germinated
speciesreg_propgerm <- lm(PropGerm ~ SpeciesName, data=blackboxsplit)
```

```
summary(speciesreg_propgerm)

#Creating D. californica Data Frame Subset
dancal <- blackboxsplit %>%
  filter(SpeciesName != "F. roemeri")

#Seed Type Regression for Proportion of Seeds Germinated
seedtypereg_propgerm <- lm(PropGerm ~ SeedType, data=dancal)
summary(seedtypereg_propgerm)

#Species Regression for Average Pathogen Species Richness
speciesreg_apsr <- lm(APSR ~ SpeciesName, data=blackboxsplit)
summary(speciesreg_apsr)

#Seed Type Regression for Average Pathogen Species Richness
seedtypereg_apsr <- lm(APSR ~ SeedType, data=blackboxsplit)
summary(seedtypereg_apsr)
```

Generalized Linear Mixed Effects Models

```{r}
#Maximum Spring Temperature Models
```

```
total.lmer3a <- lmer(PropGerm ~ SpeciesName + SeedType + SpringPPT +
SpringTmaxC + PlantDensity + LastyrElevation + APSR + (1|SiteName/PlantNr),
data=blackboxsplit)
summary(total.lmer3a)
r.squaredGLMM(total.lmer3a)
anova(total.lmer3a, type=3)

total.lmer3b <- lmer(APSR ~ SpeciesName + SeedType + SpringPPT +
SpringTmaxC + PlantDensity + LastyrElevation + (1|SiteName/PlantNr),
data=blackboxsplit)
summary(total.lmer3b)
r.squaredGLMM(total.lmer3b)
anova(total.lmer3b, type=3)

#Minimum Winter Temperature Models
```

```
total.lmer4a <- lmer(PropGerm ~ SpeciesName + SeedType + SpringPPT +
WinterTminC + PlantDensity + LastyrElevation + APSR + (1|SiteName/PlantNr),
data=blackboxsplit)
summary(total.lmer4a)
r.squaredGLMM(total.lmer4a)
anova(total.lmer4a, type=3)

total.lmer4b <- lmer(APSR ~ SpeciesName + SeedType + SpringPPT +
WinterTminC + PlantDensity + LastyrElevation + (1|SiteName/PlantNr),
data=blackboxsplit)
summary(total.lmer4b)
r.squaredGLMM(total.lmer4b)
anova(total.lmer4b, type=3)

#Soil Nitrogen Model
total.lmer5 <- lmer(PropGerm ~ SpeciesName + SeedType + SpringPPT +
LastyrElevation + SoilPercentNitrogen + PlantDensity + APSR + (1|Region/SiteName)
+ (1|SiteName/PlantNr), data=no.na.data.full)
summary(total.lmer5)
r.squaredGLMM(total.lmer5)
anova(total.lmer5, type=3)
```

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