Testing Janzen's physiological barrier hypothesis through experimental evolution and biogeographical study.

A Thesis Proposal for the degree of Doctor of Philosophy

By

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Introduction.

Diversity patterns. A major goal in ecology and evolution is to understand the factors that generate and maintain biodiversity gradients across global, continental, regional, and local scales. At broad spatial scales, many phylogenetic groups exhibit high contemporary and historic biodiversity at tropical latitudes, with generally decreasing diversity towards the poles (i.e., the latitudinal biodiversity gradient) (Hillebrand, 2004; Mittelbach et al., 2007; Pianka, 1966; Rohde 1992). A negative (but weaker) correlation between latitude and species richness is also realized at smaller (i.e., local) scales (Hillebrand, 2004). Generally, the strength (but not the direction) of the latitudinal diversity gradients differs across clades, scales, sampling grains, regions, and realms (e.g., terrestrial, freshwater, marine) (Hillebrand, 2004). For example, moth species richness decreases with latitude, but the strength of the relationship differs between the northern and southern hemispheres (Ballesteros-Mejia et al., 2016), Amphibian and mammal intraspecific genetic diversity decreases with latitude, but only significantly so above 30° North (Gratton et al., 2017), and avian species richness is higher in the tropics than in the temperate zone (Davies et al., 2007; Fjeldså et al., 2012).

Biodiversity patterns are also associated with topographical features (e.g., mountains) worldwide. Topographically complex regions display elevated levels of taxonomic and ecological diversity (Reviewed in: Badgley et al., 2017), and mountains have been implicated as important features for the creation and maintenance of biodiversity patterns. Among other factors, mountains serve as dispersal barriers and bridges, mountain building creates new habitats and changes climate patterns, climate zonation reduces extinction along montane slopes during periods of climate change by allowing species to track their preferred climate niches, and cyclical climate fluctuations over long time periods can cause montane regions to serve as species pumps through alternating periods of isolation and connectivity among similar habitats (Hoorn et al., 2013). Bird species richness patterns provide good evidence for the role of montane regions in generating and maintaining diversity: avian richness peaks correspond to major mountain chains (esp. the Andes and Himalayas) (Davies et al., 2007), avian elevational diversity patterns are shaped by both temperature and precipitation regimes (McCain, 2009a), and montane avian diversity decreases from low-latitude mountains to high-latitude mountains (Fjeldså et al., 2012).

Diversity Hypotheses. A plethora of hypotheses have been proposed as explanations for latitudinal, regional, and local diversity patterns. All hypotheses include either historical or ecological elements, however, most integrate evolutionary and ecological theory in some way (Brown, 2014). Because latitudinal biodiversity gradients have formed and dissolved many times throughout geological history (Mannion et al., 2014), effective hypotheses must be able to explain both the origination and the maintenance of diversity patterns. These explanations include, but are not limited to, hypotheses focused on speciation, extinction and/ or diversification rate, climate/ productivity, environmental/ topographical heterogeneity, environmental stability, environmental harshness, habitat arrangement/ patchiness, edaphics/ nutrients, area, biotic interactions, dispersal, history, niche conservatism/ divergence, population size, metabolic rates, growth rates, mutation rates, or some combination of these factors (reviewed in Field et al., 2009; Brown, 2014; Fine, 2015; Mittelbach et al., 2007; Pianka, 1966; Rohde, 1992; Currie et al., 2004). The specific factors that shape diversity are likely to vary across spatial (e.g., Jetz & Fine, 2012) and temporal (e.g., Pontarp & Wiens, 2017) scales and among hemispheres, continents, regions, biomes, clades, functional groups, and taxa with different life histories. For example, abiotic factors (e.g., climate) may be more important range determinants for temperate zone species than tropical species, while biotic factors (e.g., interspecific competition) may be more important in the tropics (Khaliq et al., 2017).

Climate and productivity are consistently strong predictors of variation in species/ taxonomic richness, especially at large spatial grains and extents (Field et al., 2009; Currie et al., 2004; Brown, 2014; Hawkins et al., 2003). Justifications for observed correlations between climate/ productivity and diversity patterns include, but are not limited to: (1) associations between temperature (or productivity) and mutation rates, metabolism rates, growth rates, and generation time (Ballesteros-Mejia et al., 2016; Brown, 2014); (2) associations between productivity and population size (Ballesteros-Mejia et al., 2016; Currie et al., 2004); (3) associations between long- and short-term climate variability and dispersal/ isolation, species abiotic niches, and species geographic range size (Janzen, 1967; Stevens, 1989); and (4) influences of physiological constraints, evolutionary history, niche conservatism, and niche evolution on species climate niches. Specifically, latitudinal variation in mean temperature explains species richness patterns across vertebrates (Belmaker & Jetz, 2015) and in birds (Davies et al., 2007). Contemporary primary productivity explains global species diversity patterns in sphingid moths (Ballesteros-Mejia et al., 2016), vascular plants (Gillman et al., 2015), and birds (Davies et al., 2007), as well as intra-regional diversity patterns across vertebrates (Jetz & Fine, 2012). Time-integrated primary productivity explains among-region contemporary diversity patterns in mammals, birds, reptiles, and amphibians (Jetz & Fine, 2012), as well as continental and regional patterns of mammal diversity throughout the past 20-million years (Fritz et al., 2016). Climate-associated

variation in the permeability of montane barriers, coupled with habitat heterogeneity along elevational gradients, increases opportunity for range fragmentation and population fragmentation (Badgley et al., 2016; Badgley et al., 2017), and long term climate oscillations driven by changes in Earth's orbit may allow small-ranged species (specialists, poor dispersers, narrow climate tolerances) to persist only at low latitudes, where oscillations are less pronounced, while large- ranged species (generalists, good dispersers, broad climate tolerances) can persist across latitudes (Dynesius & Jansson, 2000). Finally, species richness is associated with climate tolerance of individual species; diversity is highest in the warm and wet tropics because more physiological strategies can succeed in warm and wet conditions than in cold and dry ones (Currie et al., 2004).

A suite of complications challenge biodiversity hypothesis testing and inference. First, diversity gradient studies typically focus on a small number of variables and do not test multiple hypotheses against one another (Field et al., 2009). This practice is problematic because it can lead to inflated support for hypotheses tested in isolation and because different mechanisms or processes can drive similar diversity patterns. Studies that consider a suite of variables, test several hypotheses for observed diversity patterns, and interpret their results in the context of several hypotheses provide the best links between pattern and process (e.g., Belmaker & Jetz, 2015; Jetz & Fine, 2012). Diversity studies also tend to suffer from geographical biases that can lead to insignificant results or erroneous conclusions when patterns or processes differ in a geographically nonrandom way. Here, studies focused on European and North American biodiversity patterns are overrepresented, while studies of Asian and South American biodiversity patterns are underrepresented (Field et al., 2009). Furthermore, global hypothesis testing can obscure regional inferences if different factors underlie diversity patterns among regions. Diversity patterns and underlying processes may differ among hemispheres (Ghalambor et al., 2006) or regions (Davies et al., 2007; Hawkins et al., 2003). For example, the strongest predictors of avian species richness differ among biogeographic regions (Davies et al., 2007). Hypothesis testing at the regional scale provides the best avenue for understanding diversity patterns if regions represent 'evolutionary arenas' within which diversification and evolution has occurred relatively independently of other regions (Fine, 2015; Belmaker & Jetz, 2015; Jetz & Fine, 2012). Finally, diversity studies suffer from taxonomic biases as well. Studies that consider small clades or only species with similar life histories or functional strategies may not produce generalizable results (Fine, 2015), while studies that include many taxa, but do not consider biologically meaningful differences among them, may miss group-specific patterns and find a lack of significant associations even when such associations exist. This is because diversity patterns in different clades may be shaped by different processes (e.g., clades differ in the climate axes that most limit their spread; Ghalambor et al., 2006). Multiple clades should be analyzed in all analyses to gain a comprehensive understanding of diversity patterns and diversification processes (Fine, 2015).

Janzen's Physiological barrier hypothesis. Janzen's physiological barrier hypothesis (Janzen, 1967) provides one potential explanation for diversity patterns; the hypothesis considers the interacting influences of contemporary climate, selective history, and geography on isolation and gene flow. In Janzen's hypothesis, organisms encounter mountain passes as physiological barriers to dispersal. Janzen predicts that mountain passes will be higher (more difficult to disperse across) in the tropics than in the temperate zone for two reasons. First, low-temperature seasonality in the tropics causes pronounced thermal zonation along elevational gradients and results in little overlap in thermal regimes across elevation, while high-temperature seasonality in the temperate zone reduces elevational thermal zonation and increases thermal regime similarity. Second, low-temperature seasonality in the tropics selects for narrow thermal niche breadths in tropical organisms, while high-temperature seasonality in the temperate zone selects for broader thermal niches in temperate organisms. For these two reasons, tropical organisms are more likely to encounter mountain passes as temperature regimes to which they are not adapted (i.e., physiological barriers) than are temperate organisms (See figure I.1 for a conceptual illustration of Janzen's Mountain Pass Hypothesis). Janzen's hypothesis has been extended to predict that diversity is higher in the tropics than in the temperate zone because thermal barriers to dispersal are more difficult to cross in the low-temperature seasonality tropics than in the high-temperature seasonality temperate zone, leading to increased isolation, reduced geneflow, and increased diversification in tropical montane areas relative to temperate ones.

As described, Janzen's physiological barrier hypothesis cannot provide a general explanation for diversity patterns; it is restricted in application to montane regions with thermal barriers to dispersal. This restriction is not necessary, and clearly was not intended by Janzen. Janzen notes that the hypothesis can apply to any physiologically challenging environmental gradient. Furthermore, spatial variation in environmental variables need not be elevational, environmental variation without elevational change is just as restrictive to the movement of organisms as is elevational environmental variation. It is unclear whether thermal gradients are the main factors that cause mountains to be dispersal barriers, or if other factors that covary with elevation are also important; other climatic (e.g., precipitation) and biotic (e.g., interspecific competition) factors may influence dispersal and shape species

ranges (Ghalambor et al., 2006). Taking these points into account, researchers have applied Janzen's mountain pass hypothesis to precipitation (Kozak & Wiens, 2007; Bonetti & Wiens, 2014) and pH (Gaitan-Espitia et al., 2017; Kapsenberg et al., 2017), as well as to temperature in non-elevational contexts (Tomillo et al., 2017). Still, Janzen's mountain pass hypothesis can be made more general. Physiological barriers shouldn't just be higher in the tropics than the temperate zone; physiological barriers may restrict dispersal anywhere where there is low-environmental seasonality and/ or where species have narrow environmental niche breadths. All else equal, the diversity prediction Janzen's physiological barrier hypothesis argues that species diversity should also be high anywhere where physiological barriers to dispersal are high. There is no reason to believe that physiological barrier magnitude or species diversity are best explained by latitude, latitude is simply a convenient proxy that captures spatial variation in many measured and unmeasured biotic and abiotic variables.

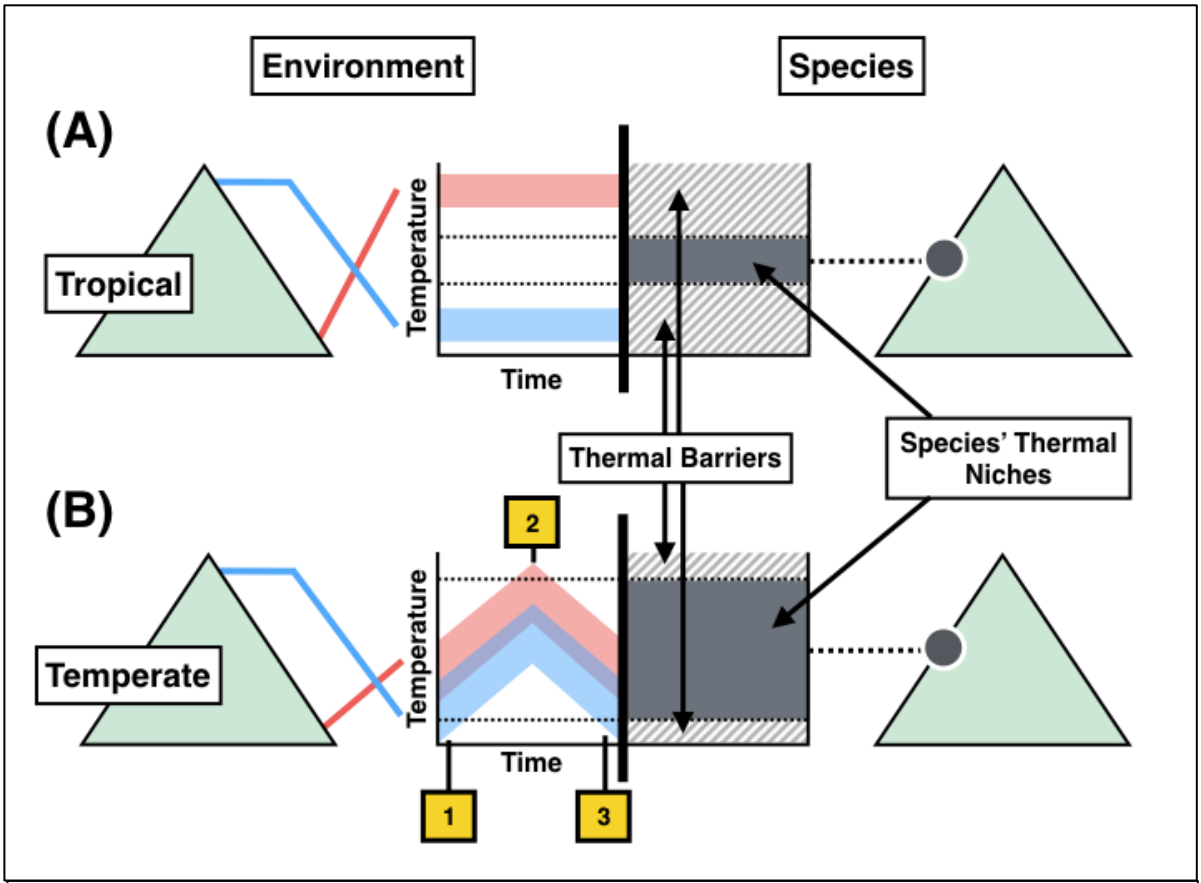


Figure I.1. Conceptual illustration of Janzen's Physiological Barrier Hypothesis. (A, Left) Annual temperature regimes for a high elevation (blue) and low elevation (red) tropical site. Temperature does not vary from month to month across the year, and there is no overlap in temperature regime between the low and high elevation sites. (A, Right) thermal niche for a mid-elevation tropical species; thermal niche (grey rectangle) is shaped by adaptation to temperature regime within species geographic range (grey circle). Any temperature beyond the thermal niche of the species acts as a dispersal barrier (grey diagonal line fill regions). The thermal niche of the tropical species does not overlap with the temperature regimes at the high or low elevation sites at any point during the year, therefore the temperature regimes at the high elevation and low elevation sites both act as dispersal barriers to this species. (B, Left) Annual temperature regimes for a high elevation (blue) and low elevation (red) temperate site. Temperature varies markedly from month to month across the year, and there is overlap (purple) in temperature regime between low and high elevation sites. (B, Right) Thermal niche for a mid-elevation temperate species; thermal niche (grey rectangle) is shaped by adaptation to temperature within species geographic range (grey circle). Any temperature beyond the thermal niche of the species acts as a dispersal barrier (grey diagonal line fill regions). The thermal niche of the temperate species does overlap with the temperature regimes at the high and low elevation sites at most points during the year, therefore the temperature regimes at the high elevation and low elevation sites both do not act as dispersal barriers at most times. However, the low elevation site becomes too hot for the mid-elevation temperate species to disperse through in the mid-summer (label: 2), while the high elevation site becomes too cold to disperse through in the mid-winter (labels: 1 & 3).

Janzen's physiological barrier hypothesis: environmental assumption. The core environmental assumption of Janzen's physiological barrier hypothesis is that temperature seasonality increases from the tropics to the poles (i.e., temperature seasonality is positively correlated with latitude) (Janzen, 1967). Evidence is generally in agreement with this assumption: Temperature seasonality is positively correlated with latitude in terrestrial environments (Janzen, 1967; Ghalambor et al., 2006) and in montane streams (Shah et al., 2017), and temperature range is positively correlated with latitude (Vázquez & Stevens, 2004). However, there are numerous factors that could cause patterns of temperature seasonality (and other measures of environmental seasonality) to deviate from the basic assumption that seasonality is positively correlated with latitude.

First, latitudinal gradients in temperature seasonality are caused mainly by variation in minimum temperature, not maximum temperature (Ghalambor et al., 2006). Second, spatial patterns of environmental seasonality need not be congruent across all environmental variables: precipitation range is negatively correlated with latitude (Vázquez & Stevens, 2004) and exhibits marked East-West variation, and it is unlikely that patterns of productivity and temperature seasonality mirror one another completely, despite the fact that net primary productivity is strongly negatively correlated with latitude in terrestrial environments (Gillman et al., 2015). It would be unreasonable to argue that the temperate zone is more environmentally seasonal than the tropics unless it is shown that (1) other environmental variables relate to latitude in the same way that temperature does, or (2) that temperature seasonality is the main variable affecting species abiotic niches and species richness patterns (Vázquez & Stevens, 2004).

Furthermore, geography and geographical features can also modify latitudinal gradients in mean temperature, temperature seasonality, and other aspects of climate. Proximity to large bodies of water with relatively stable temperatures generally mutes local/ regional temperature seasonality and increases mean temperature (Fjeldså et al., 2012; Ghalambor et al., 2006). This effect can be pronounced even at large spatial scales: the observation that terrestrial environments in the Northern hemisphere display more seasonality than those in the Southern hemisphere is best explained by increased proximity to large bodies of water in Southern hemisphere landmasses (Ghalambor et al., 2006). Topographic features (e.g., mountains) and prevailing wind patterns can shape many aspects of climate as well. Specifically, the Massenerhebung effect (mountain mass effect) reduces temperature seasonality and increases mean temperature in dense montane regions relative to regions with isolated mountain peaks or no montane features (Flenley, 1995), while wind and precipitation patterns are shaped by global-scale air currents and differ markedly between eastern and western montane slopes in areas with large mountain ranges (Lomolino et al., 2017).

Finally, geographical patterns of short-term climate variability (e.g., diurnal temperature fluctuation) do not necessarily match patterns of annual temperature seasonality (Ghalambor et al., 2006). Diurnal temperature fluctuation increases temperature variability in high-elevation, low-temperature seasonality tropical streams (Shah et al., 2017) and terrestrial environments (Ghalambor et al., 2006) relative to low-elevation, low-temperature seasonality tropical sites, and may be more pronounced in the tropics than the temperate zone overall. Diurnal temperature fluctuation also differs among microhabitats: it is more pronounced in the canopy of tropical forests than on the forest floor (Scheffers et al., 2017) and varies among pond, stream, and phytotelmata habitats (Gutiérrez-Pasquera et al., 2016).

Janzen's core environmental assumption, that temperature seasonality increases from the tropics to the poles, is a good starting point, but likely provides insufficient explanation for geographical variation in temperature seasonality, even at coarse spatial scales (Ghalambor et al., 2006; Sheldon & Tewksbury, 2014). A detailed assessment of geographic variation in temperature seasonality (and other aspects of environmental seasonality), that considers all the complicating factors described here, is necessary before the environmental assumption of Janzen's physiological barrier hypothesis can be accepted or rejected. Rejection of the basic assumption that temperature seasonality increases with latitude and explicit consideration of seasonality in other environmental variables will result in a more detailed understanding of how climate seasonality varies geographically and may provide an explanation for any deviations from the other assumptions and predictions of Janzen's physiological barrier hypothesis.

Janzen's physiological barrier hypothesis: evolutionary assumptions. The core evolutionary assumption of Janzen's mountain pass hypothesis is that species physiologies are shaped by climate seasonality such that species from low-temperature seasonality tropical environments have narrow thermal niche breadths / thermal tolerances and species from high-temperature seasonality temperate environments have wide thermal niche breadths / thermal tolerances (Janzen, 1967; Stevens, 1989). This assumption is frequently tested in two ways: [1] direct physiological measurements of performance in the field or laboratory, and [2] estimation of species Grinnellian climate niches

(*sensu.* Sobéron, 2007) as estimated from presence/absence datasets or expert range maps (see Kozak & Wiens, 2007 for an early example of approach two).

Evidence related to the assumption that species thermal niches or thermal tolerances are broader in high-temperature seasonality environments than in low-temperature seasonality environments comes in several forms and generally agrees with Janzen's reasoning. Species thermal niches/ tolerances are broader in the temperate zone than in the tropics (or are negatively correlated with latitude) for plethodontid salamanders (Kozak & Wiens, 2007), lowland amphibian tadpoles (Gutiérrez-Pasquera et al., 2016), aquatic stream insects (Shah et al., 2017), birds (Khaliq et al., 2014, Cadena et al., 2012), bats (Cadena et al., 2012), frogs (Cadena et al., 2012), lizards (Cadena et al., 2012), snakes (Cadena et al., 2012), across ectotherms (Sunday et al., 2011), and across major vertebrate clades (Cadena et al., 2012). And, thermal tolerance breadth is positively correlated with thermal seasonality in birds (Khaliq et al., 2014) and beetles (Sheldon & Tewksbury, 2014). However, thermal tolerance breadth is not related to latitude or thermal variability in mammals (Khaliq et al., 2014). Interestingly, sea turtle embryo mortality rate is more sensitive to small increases in temperature in species that dig deeper nests (cooler, less variable temperature regimes) than in species that dig shallow nests (warmer, more variable temperature regimes) and, within species, embryo mortality rate is more sensitive to small increases in temperature for nests on light sand beaches (cooler, less variable temperature regimes) than dark sand beaches (warmer, more variable temperature regimes) (Tomillo et al., 2017). Species environmental tolerances for axes other than temperature can also be broader in more variable environments, however less evidence is available for these other environmental axes. Sea urchin populations from environments with variable pH and carbon dioxide partial pressure are more tolerant to ocean acidification than those from less variable environments (Gaitan-Espitia et al., 2017), and pH sensitivity is negatively correlated with pH variability and frequency of exposure to low pH conditions in sea urchins as well (Kapsenberg et al., 2017). With respect to precipitation, there is no evidence that precipitation niche breadth is related to latitude for plethodontid salamanders (Kozak & Wiens, 2007).

Researchers also cite positive correlations between species elevational range sizes and latitude, species elevational range sizes and environmental seasonality, and species elevational range sizes and environmental niche breadth/ tolerance breadth as evidence for Janzen's evolutionary assumptions. Species elevational ranges are greater in the temperate zone than in the tropics (Ghalambor et al., 2006), with specific evidence in mayflies (Gill et al., 2016), bats (Cadena et al., 2012, McCain, 2009b), lizards (McCain, 2009b; Sheldon et al., 2015), birds, snakes, salamanders, and frogs (McCain, 2009b). However, evidence of no relationship between elevational range size and latitude exists for Plethodontid salamanders (Kozak & Wiens, 2007), frogs (Cadena et al., 2012), lizards (Cadena et al., 2012), snakes (Cadena et al., 2012), and rodents (McCain, 2009b), and evidence of a negative relationship between elevational range and latitude exists for birds (Cadena et al., 2012). Species elevational range sizes are positively correlated with temperature seasonality in Lizards (Sheldon et al., 2015) and Andean birds (Gadek et al., 2017), but elevational range is not related to precipitation regime in bats, birds, lizards, snakes, salamanders, frogs, or rodents (McCain, 2009b). Species thermal niche breadths / tolerance breadths are positively correlated with elevational range size in beetles (Shledon & Tewksbury, 2014) and lizards (Pintor et al., 2015). However, evidence of no relationship between species thermal tolerance breadth and elevational range size exists for Lizards (Sheldon et al., 2015) as well.

Additionally, researchers cite positive correlations between species geographic range sizes and latitude, species geographic range sizes and environmental seasonality, and species environmental range sizes and species thermal niche breadths / tolerance breadths as evidence for Janzen's evolutionary assumptions. Species geographic ranges are greater in the temperate zone than in the tropics for simulated species, but only when climate seasonality is included in the model (Nakazawa, 2012). Species geographic range sizes are positively correlated with temperature seasonality in sphingid moths (Grünig et al., 2017) and lizards (Pintor et al., 2015). However, species geographic range sizes are better explained by climate extremes (e.g., minimum temperature) than climate seasonality for North American trees and shrubs (Pither et al., 2003), and species geographic range sizes are not correlated with precipitation seasonality in sphingid moths (Grünig et al., 2017). Species thermal niche breadths/ tolerance breadths are positively correlated with geographic range size in lizards (Pintor et al., 2015). And, geographic range size is positively correlated with environmental tolerance breadth in a metanalysis that considers multiple taxonomic groups and axes of environmental variation (Slatyer et al., 2013). However, evidence of no relationship between species thermal tolerance breadth and geographic range size exists for birds and mammals (Khaliq et al., 2017).

As with temperature/ environmental seasonality, there are numerous factors that could cause patterns of species thermal or environmental niche breadths to deviate from the basic assumption that niche breadth is positively correlated with latitude.

First, because seasonality patterns are not congruent across all environmental axes, species niche breadths may be positively correlated across multiple environmental axes of variation or exhibit trade-offs in niche breadth among environmental axes (e.g., species with broad thermal niches could have narrow precipitation niches). Temperature and precipitation niche breadth are positively correlated in hylid frogs (Chejanovski & Wiens, 2014) and other amphibians (Bonetti & Wiens, 2014), while thermal tolerance is positively correlated with thermal variability but negatively correlated with precipitation variability in birds (Khaliq et al., 2014). There is no evidence of trade-offs between temperature and precipitation niche breadth in hylid frogs (Bonetti & Wiens, 2014) or monitor lizard (Lin & Wiens, 2017). Furthermore, experimental evolution in yeast reveals no trade-offs between salt stress tolerance and oxidative stress tolerance, despite convincing evidence that different adaptations underlie salt tolerance and oxidative stress tolerance (Dhar et al., 2012).

Geography and geographical features can modify species thermal niche breadths through thei effects on mean temperature, temperature seasonality, and other aspects of the environment. Very few studies consider proximity to large bodies of water when assessing aspects of species thermal and environmental niches. However, evidence of asymmetry in lower lethal temperatures and crystallization temperatures between northern and southern hemisphere insect species could support a role for coastal proximity in shaping species thermal niche breadths and thermal tolerances (Addo-Bediako et al., 2000). Additionally, positive correlations between latitude and geographical range size in the northern hemisphere but not the southern hemisphere also suggest a role for coastal proximity (e.g., sphingid moth range sizes decrease from north to south and are best predicted by temperature seasonality Grünig et al., 2017). Furthermore, the observations that small-ranged tropical avian species are concentrated in topographically complex coastal areas with stable thermal conditions (Fjeldså et al., 2012) and that the rate of increase in thermal tolerance breadth with latitude is greater in the northern hemisphere than the southern hemisphere in terrestrial ectothermic animals both suggest that proximity to large bodies of water shapes species thermal niches (Sunday et al., 2011).

Short-term thermal variability (e.g., diurnal temperature variation) in addition to temperature seasonality may shape species thermal niche breadths and drive physiological adaptation to thermal regimes (Ghalambor et al., 2006; Gutiérrez-Pasquera et al., 2016). Diurnal temperature fluctuation may select for increased thermal niche breadth and thermal tolerance even in thermally aseasonal environments. Diurnal temperature fluctuation causes low- and high-altitude tropical arboreal mammals, birds, reptiles, and frogs to experience similar climate regimes (Scheffers et al., 2017). However, it is unclear how diurnal temperature fluctuation translates to thermal niche breadth or thermal tolerance. Marine copepods are sensitive to small changes in mean temperature, but show little response to even wide ranges of daily temperature variability, and copepod response to diurnal temperature variation is not correlated with latitude (Hong & Shurin, 2015); this finding seems to call into question the role of diurnal temperature fluctuation in thermal adaptation.

Additionally, latitude, elevation, temperature seasonality, and mean temperature can be highly correlated (e.g., temperature seasonality and minimum annual temperature are positively correlated; (Pither et al., 2003). The structure of these correlations may impact correlations between species thermal niche breadth and latitude, elevation, or mean temperature. First, species environmental niche breadths are broader on one end of each axis of environmental variation than on the other end because environmental seasonality is correlated with mean environmental condition (Bonetti & Wiens, 2014). For example, thermal niche breadths for amphibians (Bonetti & Wiens, 2014) and monitor lizards (Lin & Wiens, 2017) are narrower in warm environments than cold environments, potentially because temperature seasonality is greater in cold environments than warm environments. And, Amphibians may have narrower precipitation niche breadths in dry climates than wet climates because precipitation seasonality reduced in dry climates relative to wet climates (Bonetti & Wiens, 2014), however there is no relationship between annual precipitation and precipitation niche breadth in monitor lizards (Lin & Wiens, 2017). Second, species thermal niches can be broad at high elevations and narrow at low elevations because temperature variability, driven by diurnal temperature fluctuations, is greater at high elevations than low elevations, especially in the tropics (Ghalambor et al., 2006). Evidence that supports this correlation is found in Andean frogs, where a marginally non-significant positive correlation between elevation and thermal niche breadth is observed (Von May et al., 2017). However, studies that relate elevation to elevational range find mixed support: elevational ranges are larger for high-elevation than low-elevation lizards (Sheldon et al., 2015) and vertebrates (McCain, 2009b), but no strong correlation between elevation and elevational range size is found for vertebrates, invertebrates, and plants in another study (McCain & Knight, 2013). Positive correlations between elevational range size and latitude are stronger for low-elevation than high-elevation vertebrate species (McCain, 2009b), but observations that there is no correlation between elevational range size and diurnal temperature variation across terrestrial vertebrates (Qian et al., 2017) make these findings hard to interpret. Finally, geographical variation in species thermal niches can be asymmetrical if maximum temperature or minimum temperature vary asymmetrically with latitude or elevation

(Ghalambor et al., 2006). Terrestrial temperate organisms have broader thermal tolerances mainly through increased tolerance to cold temperatures; there is less latitudinal variation in heat tolerance (Ghalambor et al., 2006; Sunday et al., 2011). For example, increased thermal niche breadth in temperate tadpoles (Gutiérrez-Pasquera et al., 2016), insects (Addo-Bediako et al., 2000), birds, and mammals (Khaliq et al., 2014) is driven by decreases in cold tolerance and cold temperatures constrain species ranges more than warm temperatures in temperate regions (Pither et al., 2003; Khaliq et al., 2017). Elevational patterns are more complex: thermal tolerance breadth in aquatic stream insects declines with elevation in the temperate zone because heat tolerance declines with increasing elevation while cold tolerance does not vary with elevation; thermal tolerance breadth increase with elevation for stream insects in the tropics because heat tolerance declines slower than cold tolerance with increasing elevation. More straightforward patterns are found in ants: ants exhibit less variation in upper than lower thermal limits across African mountain slopes (Bishop et al., 2017).

Species' environmental niche breadths may be broader in benign (hot/ wet/ productive) environments or in harsh (cold/ dry/ unproductive) environments. Environmental niche breadths can be broader in benign than harsh environments because of filtering or specialization in harsh environments and/ or because more physiological strategies can survive in warm/wet than cold/dry environments (Currie et al. 2004). Amphibian precipitation niche breadths follow this pattern: precipitation niches are narrower in dry than wet environments because special adaptations are required for amphibians to survive in dry climates (Bonetti & Wiens 2014). On the other hand, environmental niche breadths can be broader in harsh than benign environments because species expanded from ancestral benign environments to environments with harsh climates by acquiring traits that allowed them to survive harsh conditions (Sunday et al., 2011; Qian & Sandel, 2017). Evolutionary conservatism of heat tolerance and plasticity in cold tolerance of lowland amphibian tadpoles (Gutiérrez-Pasquera et al., 2016), narrower climate niche breadths in hot/ wet environments than cold/dry environments in Hylid frogs (Chejanovski & Wiens, 2014), and the fact that the geographic ranges of most bird and mammal species are centered on hot/ wet conditions (Boucher-Lalonde et al., 2016) support this prediction.

There is also a chance that species environmental niche breadths are not correlated with latitude or elevation; species can adapt to environmental conditions by shifting their environmental niche, changing the shape of their performance-environment functions, or via increased performance in optimal environments (Hong & Shurin, 2015). Evidence for this prediction is limited: Andean frogs decrease both their maximum and minimum thermal tolerance with elevation (von May et al., 2017), and marine copepods shift their thermal niches towards colder temperatures at high latitudes, but display no difference in thermal niche breadth, despite positive correlations between thermal seasonality and latitude (Hong & Shurin, 2015). Additionally, marine copepod fitness at thermal optimum is positively correlated with latitude; higher latitude populations have higher fitness peaks at their thermal optimums than lower latitude populations (Hong & Shurin, 2015). Finally, pond snails from cold, seasonal, and warm habitats have the same environmental temperature preferences, and trade-offs between acute and chronic thermal tolerance are observed for warm environment pond snails, but no difference in thermal tolerances between cold and seasonal environment snail populations is observed (Johansson & Laurila, 2017).

Species that avoid climate extremes (e.g., through seasonal migration, hibernation, or habitat use) should have narrower environmental niche breadths than species that experience the full range of environmental seasonality in a location (Ghalambor et al., 2006). In support of this prediction, ground dwelling tropical vertebrates experience a narrower range of climate seasonality than canopy dwelling species (Scheffers et al., 2017) and macro- and microclimate are both important predictors of thermal tolerance range in amphibian tadpoles (Gutiérrez-Pasquera et al., 2016). Evidence that thermoregulatory behavior can decouple body temperature from environmental temperature supports this prediction as well (Sunday et al., 2011) and suggests that it may be important to consider body temperature rather than air temperature when calculating species thermal regimes (Ghalambor et al., 2006; Buckley et al., 2013). Furthermore, realized temperature seasonality (temperature seasonality during active periods only) is a better predictor of thermal tolerance than annual temperature seasonality in beetles (Sheldon & Tewksbury, 2014); this could be true for other taxa as well. On a related topic environmental tolerance breadth may differ across life stages; environment may impact early life stages or dispersing propagules differently than adult organisms (Sheldon et al., 2015; Tomillo et al., 2017).

The core assumption, that species thermal niches increase in breadth from the tropics to the poles, is a good starting point, but this assumption undoubtedly provides and insufficient explanation for variation in species thermal and environmental niche breadths. A detailed evaluation of geographic variation in the breadth of species thermal niches that considered multiples taxonomic groups (Ghalambor et al., 2006; Khaliq et al., 2014; McCain, 2009b), deviations from the basic assumption that temperature seasonality is positively correlated with latitude (Ghalambor et al., 2006), and that incorporates information about species phylogenetic- and life-histories (Ghalambor et al., 2006) is necessary before the evolutionary assumptions of the physiological barrier hypothesis can be accepted or

rejected. Furthermore, direct evidence that fluctuating environmental conditions select for broad niches while uniform environmental conditions select for narrow niches is necessary. This evidence is required to link the proposed evolutionary mechanisms of the physiological barrier hypothesis to observed patterns of species physiological niches/ tolerances. Rejection of the basic assumption that thermal niche breadth is positively correlated with latitude coupled with study of other axes of species environmental niches will result in a more accurate understanding of how thermal niche breadth and environmental niche breadth varies geographically. A more detailed understanding of how species environmental niche breadths are shaped by selective history will either provide credibility to the mechanisms proposed by Janzen in his physiological barrier hypothesis or highlight other aspects of selective history that may be important determinants of species environmental niche breadths and physiological tolerances.

Janzen's physiological barrier hypothesis: thermal barrier prediction. The core prediction of Janzen's physiological barrier hypothesis is that thermal barriers are higher in the tropics than in the temperature zone (i.e., thermal barrier magnitude is negatively correlated with latitude). Here, barrier magnitude is shaped by both the degree of overlap in temperature regime between environments across the year and by the interaction of species thermal niches with contemporary temperature regimes. Studies that assess barrier magnitude do so in a variety of ways, however, there are relatively few studies overall that explicitly consider the thermal barrier predictions of Janzen's physiological hypothesis; most studies focus on variation in species thermal tolerances or thermal niche breadths. Thermal barriers measured using climate overlap as a metric of barrier magnitude find general support for Janzen's predictions. thermal barriers between low and high elevation terrestrial sites are generally larger in the tropics than in the temperate zone (Zuloaga & Kerr, 2016; Buckley et al., 2013), although exceptions exist (Zuloaga & Kerr, 2016). Additionally, overlap in thermal regimes across elevation are greater for temperate streams than tropical streams (Shah et al., 2017). Thermal barriers have also been measured using dispersal or gene flow to quantify barrier magnitude and evidence generally agrees with the prediction that dispersal along and across tropical mountains is reduced relative to temperate mountains (Ghalambor et al., 2006). A recent study directly measured gene flow and dispersal distance and found that mountain passes restrict dispersal and gene flow of co-distributed amphibian species. Here, direct measures of individual dispersal distance match genetically quantified patterns of gene-flow and population connectivity (Sánchez-Montes et al., 2017). Species assemblage dissimilarity and faunal turnover across space have also been used to quantify thermal barrier magnitude. Altitudinal faunal turnover is greater in the tropics than the temperate zone (Ghalambor et al., 2006), and both mammal and amphibian species assemblage similarity decrease with thermal barrier magnitude such that large thermal barriers are associated with lower species assemblage similarity (Zuloaga & Kerr, 2016). Variation in thermal barrier magnitude may also be associated with patterns of range filling such that range filling is decreased when large thermal barriers that restrict the dispersal of organisms are present in the environment. Spatially explicit ecological simulations produce species with greater range filling in the temperate zone relative to the tropics and montane regions only when temperature seasonality is included in the models (Nakazawa, 2012). Finally, variation in thermal barrier magnitude is likely to underlie variation in diversification rates between thermal specialists and thermal generalists. Bird, mammal, and amphibian thermal specialists (with narrow thermal niches / thermal tolerance breadths) have higher net diversification and speciation rates and lower extinction rates than thermal generalists (with wide thermal niches / thermal tolerance breadths) (Rolland & Salamin, 2016). Deviations from the basic assumption that temperature seasonality is positively correlated with latitude may directly or indirectly (via the effect of temperature seasonality on the evolution of species thermal niche breadths) impact geographical variation in thermal barrier magnitude. Diurnal temperature fluctuation can increase thermal overlap among locations and select for broader thermal niches. The potential for diurnal temperature fluctuation to mediate barrier magnitude is illustrated by the fact that temperature regimes overlap more between low- and high-altitude tropical canopy environments than between low- and high-altitude tropical forest floor environments (Scheffers et al., 2017), and by the fact that these differences in thermal overlap cause physiological barriers across elevationally separated sites to be smaller for canopy than forest floor species (Scheffers et al., 2017). Geography can also mediate thermal barrier magnitude. Strong thermal-elevational barriers are not restricted to the tropics; high thermal barriers occur in the North-Western regions of North America as well (Zuloaga & Kerr, 2016; Currie, 2017) and are associated with proximity to large bodies of water, namely the pacific ocean. Geographical factors may cause thermal barrier magnitude to be higher for high-elevation species than low-elevation species as well. This is because low-elevation species can filter around high-elevation barriers, but high-elevation species must generally disperse across low-elevation sites to reach isolated high-elevation environments (Zuloaga & Kerr, 2016). Phylogenetic history, life history, and alternative strategies of climate adaptation can also shape thermal barrier magnitude. Thermal barrier magnitudes are smaller for mammals than amphibians, possibly due to homeothermy and increased dispersal potential in mammals compared to amphibians

(Zuloaga & Kerr, 2016). Differences in thermal barrier magnitude among co-distributed amphibian species are also thought to be shaped by specific life history traits, dispersal potentials, and breeding strategies (Sánchez-Montes et al., 2017). Finally, elevational gradients may present less of a barrier to dispersal and gene flow among populations at different elevations along a single slope for avian elevational migrants than for more sedentary avian species (Gadek et al., 2017).

The core prediction that thermal barrier magnitude decreases from the tropics to the poles is a good starting point, but likely provides insufficient description of geographical variation in thermal and environmental barrier magnitude. Deviations from the environmental seasonality and evolutionary assumptions of Janzen's physiological barrier hypothesis, and evidence from studies that directly examine some aspect of thermal barrier magnitude, indicate that latitude cannot be used as a surrogate for thermal barrier magnitude. A detailed assessment of geographic variation in thermal and environmental barrier magnitude, that considers all the complicating abiotic and biotic factors, is necessary before the barrier predictions of Janzen's hypothesis can be accepted, modified, or rejected. Rejection or partial rejection of the basic assumption that thermal barrier magnitude is negatively correlated with latitude will result in a better understanding of how thermal barrier magnitude varies geographically and is affected by variation in temperature seasonality and species thermal niche breadths, and will provide justification for study of other environmentally derived physiological barriers to dispersal (e.g., aridity barriers).

Janzen's physiological barrier hypothesis: diversity prediction. Janzen's physiological barrier hypothesis has been proposed as a potential explanation for latitudinal biodiversity patterns. Specifically, species richness should be negatively correlated with latitude because increased thermal barrier magnitude in the tropics results in increased isolation and accumulation of species relative to the temperate zone, where thermal barriers are less effective at isolating populations and restricting gene flow. Evidence supporting Janzen's physiological barrier hypothesis as an explanation for contemporary species richness patterns can come in several forms that differ in the degree of support that they provide to the hypothesis. The strongest evidence for Janzen's hypothesis would be provided by a negative correlation between species richness and physiological barrier magnitude. To my knowledge, no strong tests of this relationship have been carried out. However, some evidence suggests that there is not always a negative correlation between species richness and physiological barrier magnitude. Thermal barriers, quantified using temperature regime overlap, are high in the neotropics and on the north-western regions of North America around 55°N (Zuloaga & Kerr, 2016). However, there is no evidence of species richness peaks in the North American regions identified to have high thermal barriers to dispersal (Zuloaga & Kerr, 2016; Currie, 2017). The next strongest support for Janzen's hypothesis as an explanation for biodiversity patterns would come from both a positive correlation between temperature seasonality and latitude *and* a positive correlation between species thermal niche breadths and latitude. Here, evidence is supportive: higher cryptic species richness is found in tropical mayfly assemblages that experience low-temperature seasonality and have narrow elevational ranges than in temperate mayfly assemblages that experience high-temperature seasonality and have larger elevational ranges (Gill et al., 2016). Next, negative correlations between temperature seasonality and species richness (or other diversity measures) provide relatively ambiguous support for the hypothesis. Supporting evidence comes from a globally negative correlation between temperature seasonality and species richness in sphingid moths (Ballesteros-Mejia et al., 2016). However, seasonality may limit the spread of tropical groups into more temperate regions rather than impacting isolation and barrier magnitude within regions (Ballesteros-Mejia et al., 2016; Chejanovski & Wiens, 2014). Additionally, high taxonomic diversity in fossil communities and high beta-diversity in fossil taxonomic diversity in low-temperature seasonality mid-latitude Eocene uplands supports a positive correlation between temperature seasonality and species richness that is decoupled from latitude (Archibald et al., 2013). Finally, a negative correlation between species thermal niche breadth and species richness provides ambiguous support for the hypothesis as well. Despite ambiguity in interpretation, evidence is generally supportive: richness and environmental niche breadth are negatively correlated for birds and mammals globally (Boucher-Lalonde et al., 2016), high tropical montane avian diversity is driven by the aggregation of small-ranged species in topographically complex regions (Fjeldså et al., 2012), and hylid frogs in high richness climates tend to exhibit narrow thermal and precipitation niche breadths (Chejanovski & Wiens, 2014). Furthermore, richness and abiotic niche width are negatively correlated in an evolutionary simulation study (Pontarp & Wiens, 2017). Complications and complexities abound in studies that attempt to explain diversity patterns. One such complication that is particularly relevant here is that species richness may not be the ideal diversity measure; the physiological barrier hypothesis may apply better to major clades, genera, or subspecies than it does to taxonomically distinct species (e.g., mayfly cryptic species diversity conforms to the diversity predictions of Janzen's hypothesis, but mayfly species identified taxonomically do not; Gill et al., 2016).

The diversity prediction of Janzen's extended physiological barrier hypothesis requires more testing before any solid conclusions can be drawn. However, current evidence is supportive for the most part. It will be valuable to assess all correlates of the diversity predictions across clades, life history strategies, regions, and environmental variables. This detailed assessment of geographic variation in species richness will provide the first step towards accepting or rejecting Janzen's physiological barrier hypothesis as an explanation for latitudinal and large-scale species richness patterns.

Conclusion. For my thesis research, I propose a multifaceted evaluation of Janzen's physiological barrier hypothesis using experimental evolution and comparative phylogenetic study. I focus my proposal on (1) testing the evolutionary mechanisms underlying Janzen's assumption that selective history shapes physiological tolerances and, in turn, shapes the effectiveness of physiological barriers to dispersal, (2) describing geographical and biological variation in the magnitude of physiological barriers that separate sister species, and (3) assessing to what degree Janzen's hypothesis can account for latitudinal variation in species richness. In Aim 1 I develop a yeast experimental evolution system amenable to testing evolutionary theory in a controlled experimental environment. I use this system in Aim 2A to evolve yeast with selective histories in environments with uniform or fluctuating stress concentration. In Aim 2B I propose to study how selective history in uniform versus fluctuating environmental conditions impacts yeast survival and reproductive capacity in subsequent exposure to physiological challenges that can act as dispersal barriers; I also propose a more general evaluation of adaptation to constant versus fluctuating conditions. In Aim 3, I propose to use biogeographical and comparative phylogenetic techniques to evaluate whether or not thermal barriers to dispersal actually decrease in magnitude from the tropics to the temperate zone by measuring the sizes of thermal barriers that separate sister species. I also propose a biodiversity assessment in Aim 3 to determine whether Janzen's physiological barrier hypothesis can explain global patterns of extant vertebrate species richness. Finally, I plan to consider major biological and abiotic factors known cause observed patterns of climate seasonality, species physiological niche/ tolerance breadths, and physiological barrier magnitude to deviate from Janzen's predictions in all analyses throughout Aim 3, and I plan to repeat all analyses from Aim 3 using additional environmental variables (e.g., productivity and precipitation) in order to assess the generality of Janzen's Physiological barrier hypothesis as an explanation for recent speciation events as well as global biodiversity patterns.

Aim 1: Experimental Evolution System

Summary. In this aim of my thesis, I troubleshoot, refine, and validate a novel yeast experimental evolution system for testing evolutionary theory in a controlled experimental environment (see Lenski et al. 1991 for an early example of the utility of experimental evolution). Experimental evolution studies are now regularly used to evaluate ecological and evolutionary theory; they are particularly useful for studies of adaptation to environmental conditions, including studies of evolutionary rescue (Gonzalez & Bell, 2013), adaptation to gradual and abrupt environmental change (Gorter et al., 2016, 2017), genetic variation (Lenski et al., 1991; Cooper & Lenski 2010), and maintenance of physiological strategy heterogeneity within wild populations (Holland et al. 2014). My experimental evolution starting material is approximately 200 haploid and 200 diploid yeast strains, each of which can be identified by a unique 19-20 base pair barcode inserted into the HO promoter. All of the 200 haploid and 200 diploid yeast strains originate from two, unbarcoded, haploid laboratory strains of *Saccharomyces cerevisiae,* and barcode insertion has been previously confirmed for all haploid and diploid strains in our libary. The use of a relatively small number of carefully inventoried barcoded strains contrasts with typical experimental evolution systems in which thousands of unconfirmed barcoded strains or just a handful of antibiotic resistant/ fluorescently tagged strains are used. These other techniques have their own strengths and weaknesses, but they are not ideal for all experimental evolution applications. Our inventoried, barcoded system allows me to precisely manipulate the starting diversity of my experimental evolution trials, monitor changes in barcode composition through time, calculate fitness changes for each strain individually, accurately repeat or restart evolutionary trials at any time using the same starting material, and multiplex my DNA extraction and sequencing samples to increase throughput and reduce costs for all experimental evolution and competition assays. Before applying this experimental evolution system to test evolutionary theory, it is first necessary to (1) Explore, perfect, and standardize appropriate experimental and sample processing workflows, (2) confirm our ability to distinguish yeast strains by barcode identity using Ion Torrent sequencing, (3) assess the utility of Ion Torrent sequencing as a community census tool in this system, (4) test the utility of a sequencing-based competition assay as a method to detect changes in yeast fitness, (5) confirm that barcode insertion did not create fitness differences among yeast strains with different barcode sequences, and (6) explore differences in evolutionary dynamics between haploid and diploid yeast (evolutionary speed differs with ploidy: Gerstein et al., 2010; Selmecki et al. 2015), differences based on starting culture concentration, and differences associated with evolution in the presence of chemical stressors (adaptation to chemical stress can increase evolved lethal limits relative to ancestral lethal limits (Gonzalez & Bell, 2013), and mechanisms of adaptation to different chemical stressors may vary (Gorter et al., 2016, 2017)). I address the first three points in Aim 1A using a thirty-generation growth assay and the latter three points in Aim 1B using a 250-generation experimental evolution trial paired with a sequencing-based competition assay. The data generated for Aim 1 are largely preliminary: experimental evolution, competition assay, and sample processing methods are fairly heterogeneous throughout Aim 1 and some errors in competition assay design (Aim 1B) complicate analysis of the results from this assay. To alleviate these concerns, I plan to also include a subset of the competition assay sequencing data from Aim 2 in my analyses of initial fitness differences among barcoded yeast strains (4, above) and changes in yeast fitness over the course of experimental evolution (5, above).

Aim 1A: Initial attempt at yeast growth assays, sample processing, and Ion Torrent sequencing. Before testing anything in this novel yeast experimental evolution system, it was necessary to (1) explore, troubleshoot, and standardize appropriate experimental and sample processing workflows, (2) confirm our ability to distinguish yeast strains by barcode identity using Ion Torrent sequencing, and (3) assess the utility of Ion Torrent sequencing as a community census tool in this system.

Growth Assay. To address the goals of this aim, I revived yeast from freezer stock, combined them in groups of 1, 2, or 92, and grew them in liquid media for three transfers (30 generations). I collected yeast samples for DNA extraction, processing, and DNA sequencing from all wells after the first transfer (generation 10) and again after the third transfer (generation 30). See Table 1.1 for experimental design summary information.

(A)	# experimental wells	strains/ experiment	Ploidy	Growth Medium	Dilution
Treatment 1	10	92	Diploid	CM	1:1000
Treatment 2	30	2	Diploid	CM	1:1000
Treatment 3	10	1	Diploid	CM	1:1000

(B)	Transfer
DNA Samples	1, 3
Stock Plates	1, 3

Table 1.1 Experimental design for Aim 1A. (a) treatment information for growth assay; number of experimental wells per treatment, number of strains per experimental well, yeast ploidy, growth medium, and inoculation dilution. (b) Collection time points for DNA samples and -80°C stock plates.

Revive Yeast. I revived yeast from single-colony derived cultures in 96-well shallow well microplate stocks stored at -80° Celsius. Each stock-microplate well contained 100ul overnight yeast culture plus 100ul 30% glycerol in H_2O. To revive the yeast, I thawed the stock microplates and then thoroughly resuspended (mixed) the yeast in each well using a pipette. I removed 20ul of sample from each well of the thawed stock microplate and transferred this volume to the corresponding well (same plate position) of a 96-well deep well microplate containing 480ul of complete minimal media per well (1:25 dilution). I then covered the deep well microplate with a breathable membrane and incubated it for 24 hours at 30° Celsius. Following incubation, I diluted yeast 1:20 in a fresh deep-well microplate containing 500ul compete minimal media, and incubated the plate for an additional 24 hours at 30° Celsius. The 24-hour culture from this second transfer served as the starting material for the liquid growth assay.

Liquid Growth Assay. I mixed revived yeast strains in sets of 1, 2, or 92 according to the Aim 1A. experimental design (Table 1.1) prior to the start of the liquid growth assay. After mixing, I diluted the yeast 1:1000 using a two-step dilution in complete minimal media (CM). I added 500ul of the 1:1000 diluted yeast mixtures to the corresponding wells of a 96-well deep well microplate and incubated the microplate for 24 hours at 30° Celsius. After 24 hours of growth, I removed a 60ul yeast sample for DNA extraction from each well (hereafter referred to as the generation-10 sample), and I again diluted the overnight culture yeast 1:1000 in a 96-well deep well microplate containing 500ul fresh CM. I completed this process a third time, for a total of three transfers. I collected a second 60ul yeast sample for DNA extraction following 24 hours of growth in the final deep well microplate (hereafter referred to as the generation-30 sample).

Sample Processing. I extracted DNA from Generation-10 and generation-30 yeast samples using three DNA extraction protocols ((1) Zymo Scientific Fungal/Bacterial DNA Miniprep kit, (2) homebrew Lyticase + Beads protocol, and (3) homebrew SDS protocol). I quantified DNA concentration prior to downstream processing using two complimentary methods. First, I measured DNA concentration using a Qubit High Sensitivity DNA Quantification Kit and a Qubit 3.0 Flourometer. I then confirmed the measured concentrations by running a 1% Agarose gel and comparing sample band intensity to Flourometer concentration data. I subsequently attached a unique combination of forward and reverse Ion Torrent sequencing primers to the DNA fragments in each sample using a thermocycler (94°C for 5 minutes; 25 cycles - 94°C for 10 seconds, 50°C for 10 seconds, 72°C for 10 seconds; 72°C for 5 minutes). I measured PCR product DNA

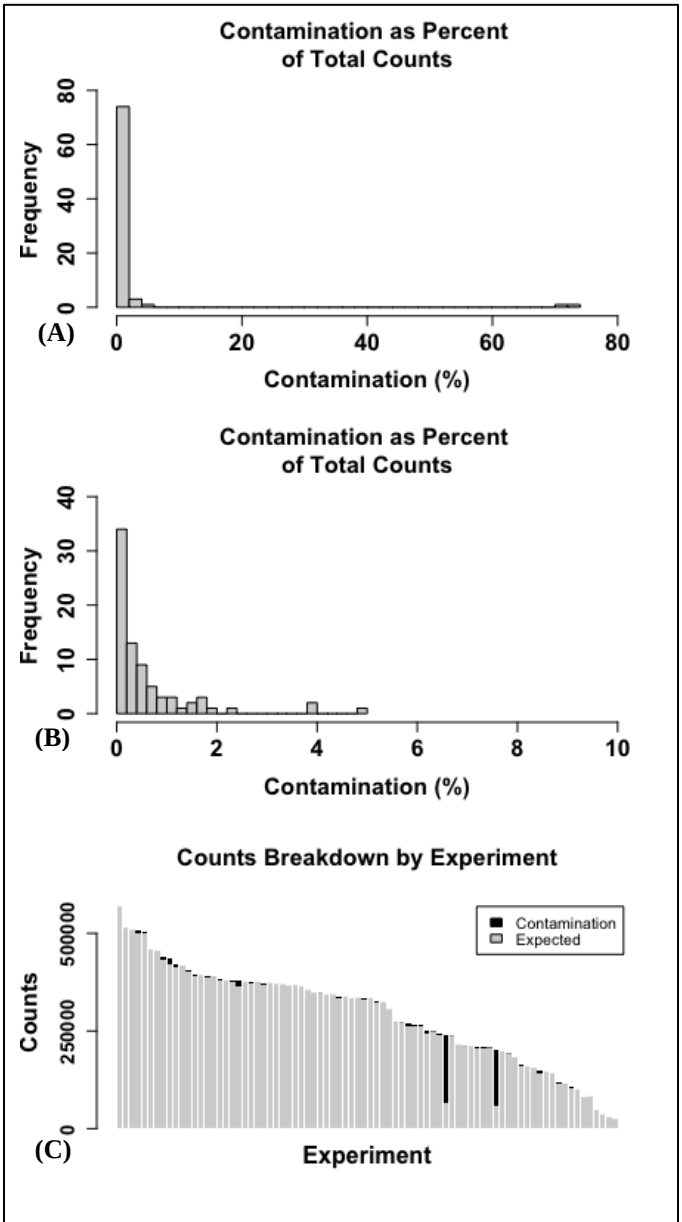


Figure 1.1 Contamination and counts summary information for Aim 1A sequence data processing. (A) Percent contamination by experiment for all experiments in the sequencing run. (B) Percent contamination by experiment for all experiments with less than 10% contamination. (C) Total counts recovered for all experiments; bars are broken to show distribution of expected and contamination counts for each experiment. Note that only those experiments for which contamination detection was possible are included in this figure. Contamination percent cannot be estimated for experiments containing all 92 barcodes. Figure produced in R (R Core Team, 2017).

concentrations using the Qubit Flourometer (described above) and confirmed these measurements using a 1% Agarose gel. I visually inspected agarose gel bands to ensure that each sample produced a single clean band at the desired fragment length. Some samples did not reach the desired DNA concentration following 25 cycles of PCR; I ran a second aliquot of the extracted DNA for these samples through the thermocycler using the same primer addition protocol, but with additional PCR cycles (either 30 or 35 total cycles vs. the original 25). Once all samples met the desired PCR product DNA concentration and passed visual inspection on an agarose gel, I pooled the samples (into several pools) based on their DNA concentrations and purified them using a DNA Clean & Concentrator kit from Zymo Scientific. I again measured DNA concentration using the Qubit Flourometer, and I then pooled the pooled samples according to their DNA concentration to form a final Library for DNA sequencing. Finally, I delivered the purified library to the Dorsett Lab at Saint Louis University for sequencing. There, DNA concentration and fragment size were quantified using a GC Trace (Agilent DNA 1000) prior to Ion Torrent sequencing. Ion Torrent sequencing was performed according to standard protocols.

Sequence Data Processing. Ion Torrent sequencing recovered 115,308,578 library ion sphere particles. 42.8% of these reads were polyclonal, and 19.1% were low read quality, resulting in a final library of 44,888,609 high-quality reads (39% of total). I received sequence data in the form of fastQ files. I processed all fastQ files using R to create a read database containing 33,154,811 reads that perfectly matched our expected fragment sequences (forward primer, reverse primer, and yeast barcode sequence were all required to match our records exactly). I assigned the perfectly matched reads to experiments (growth assay microplate wells) by forward and reverse barcode identity, and to yeast strains within each experiment by yeast barcode sequence. I used R to convert these assigned reads to counts in order to determine the number of counts recovered for each yeast strain in each of the experiments. This process ultimately resulted in 28,974,198 counts that mapped to the correct yeast strains and experiment identities. In some instances I detected yeast strains in experiments where they should not have been present; a total of 441,417 counts fall within this category. I used these counts to assess the distribution of contamination rates among experimental wells (Fig. 1.1) Note that 20 experiments included in the design included all 92 yeast strains; these experiments appear to have 0.0% contamination when analyzed in the same way as the other experiments, these experiments are not included in the figures unless otherwise noted. From these counts data, I produced a counts matrix (yeast strain by row, experiment identity by column) that I will use for all subsequent analyses.

Future Work. The entirety of the wet-lab and sequence data processing work is complete for Aim 1A. I have visually inspected the data and produced preliminary plots depicting the change in barcode proportion over the 30-generation growth assay. I can anecdotally confirm our ability to distinguish yeast strains by barcode identity using Ion Torrent sequencing (goal (2) for Aim 1), as well as the utility of Ion Torrent sequencing as a community census tool (goal (3) for Aim 1). However, I have not yet performed explicit statistical analyses to confirm these results. The growth assay and sample processing performed in Aim 1A allowed me to explore and troubleshoot many aspects of the experimental evolution system; these experiences informed my techniques and methodology for Aim 1B (partial accomplishment of goal (1) for Aim 1).

Aim 1B: Experimental evolution and sequencing-based competition assay pilot. After our first pilot study provided promising preliminary results, it was necessary to (4) test the utility of a sequencing-based competition assay as a method to detect changes in yeast fitness, (5) confirm that barcode insertion did not create fitness differences among yeast strains with different barcode sequences, and (6) explore differences in evolutionary dynamics between haploid and diploid yeast, differences based on starting culture concentration, and differences associated with evolution in the presence of chemical stressors. Preliminary results from Aim 1B are presented in Figure 1.2.

Experimental Evolution. To address the goals of this aim, I revived haploid and diploid yeast from freezer stock, combined them in groups of 2, 4, 8, or 12, and grew them in liquid media for twenty-five transfers (250 generations). Diploid yeast pairs were inoculated at three concentrations (1:1000, 1:250, and 1:4000) in complete minimal media (CM); a second set of diploid yeast pairs were inoculated at standard concentration (1:1000) in CM plus ethanol, and CM plus NaCl. I inoculated haploid yeast pairs at the standard 1:1000 dilution in CM for comparison against diploid pairs exposed to the same selective conditions. I collected yeast samples for DNA extraction, processing, and DNA sequencing from all wells after the first (generation-10), 10^{th} (generation-100), 15^{th} (generation-150), 20^{th} (generation-200), 22^{nd} (generation-220), 24^{th} (generation-240), and 25^{th} (generation-250) transfers. I created -80°C yeast stock plates at each of these seven time points as well. See Table 1.2 for experimental design summary information.

(A)	# experimental wells	strains/ experiment	Ploidy	Growth medium	Dilution
Treatment 1	11	2	Haploid	CM	1:1000
Treatment 2	22	2	Diploid	CM	1:1000
Treatment 3	11	2	Diploid	CM + Ethanol	1:1000
Treatment 4	11	2	Diploid	CM + NaCl	1:1000
Treatment 5	11	2	Diploid	CM	1:250
Treatment 6	11	2	Diploid	CM	1:4000
Treatment 7	5	4	Diploid	CM	1:1000
Treatment 8	4	8	Diploid	CM	1:1000
Treatment 9	2	12	Diploid	CM	1:1000

(B)	Transfer
Evolution DNA Samples	1, 10, 15, 20, 22, 24, 25
Competition Assay Material	1, 25
Stock Plates	1, 10, 15, 20, 22, 24, 25

Table 1.2. Experimental Design for Aim 1B. (A) Treatment information for experimental evolution assay: number of experimental wells per treatment, number of strains per experimental well, yeast ploidy, growth medium, and inoculation dilution. (B) Collection time points for experimental evolution DNA samples and -80°C stock plates as well as starting material for competition assays.

Revive Yeast. I followed the protocol described in Aim 1A section "Revive Yeast" to revive the yeast for this experiment. Here, frozen yeast stocks were only allowed one 24-hour period of growth in CM (1:20 initial dilution) before beginning the experimental evolution assay (as opposed to the two 24-hour growth periods in Aim 1A).

Experimental Evolution Assay. I mixed revived yeast strains in sets of 2, 4, 8, and 12 prior to the start of the experimental evolution assay. All treatments called for sets of 2 yeast strains per well other than the 11 wells in treatments 7,8, and 9 of the experimental design, which called for sets of 4, 8, or 12 yeast strains, respectively. These treatments were included in the design with the express goal of exploring differences in our ability to detect evolutionary dynamics based on the number of strains present. Experimental evolution treatments were as follows: treatment 1, haploid yeast pairs in CM; treatment 2, diploid yeast pairs in CM; treatment 3, diploid yeast pairs in CM + Ethanol; treatment 4, diploid yeast pairs in CM + NaCl; treatment 5, diploid yeast pairs inoculated at 4x standard dilution; treatment 6, diploid yeast pairs inoculated at 1/4x standard dilution; treatments 7, diploid yeast sets of 4 in CM; treatment 8, diploid yeast sets of 8 in CM; and treatment 9, diploid yeast sets of 12 in CM. After combining pairs and sets of yeast strains, I diluted the yeast populations according to the experimental design using a two-step dilution in CM (or CM plus either ethanol or NaCl). I arrayed these diluted yeast mixtures in a 96-well deep well microplate according to the experimental design and incubated the microplate for 24 hours at 30°C. After 24 hours of growth, I removed a 60ul yeast sample for DNA extraction from each well (generation-10 sample), created a generation-10 -80°C stock microplate, diluted the 24-hour cultured yeast, and arrayed the diluted yeast in a fresh deep well microplate, using the same media type and dilution arrangement as described for the initial set up. I transferred the yeast populations in this way every 24 hours until 25 transfers were completed. I collected additional samples for DNA extraction and created -80°C stock microplates after the 10^{th}, 15^{th}, 20^{th}, 22^{nd}, 24^{th}, and 25^{th} transfers. Freezer stock plates provide me with a means to revive yeast from the time points of interest for future competition assays, and also serve as backup copies of evolving populations that provide a means to restart the experimental evolution from any time point in which a stock microplate was created.

Sequencing-based competition. I used sequencing-based competition assays to quantify 1) differences in fitness among ancestral (generation-10) yeast, indicative of effects of barcode insertion on yeast fitness, and 2) changes in yeast fitness associated with 250 generations of evolution in the experimental evolution treatment conditions described. In these assays, I competed evolved (generation-250) and ancestral (generation-10) yeast against a common reference strain in complete minimal media (CM). I measured the fitness of the ancestral and evolved yeast relative to the reference strain, and I quantified the change in fitness for each yeast strain over the 240 generations of experimental evolution.

Sequencing-based competition assay. To conduct the competition assays, I revived reference yeast, generation-10 yeast, and generation-250 yeast according to the protocol outlined in the "revive yeast" section of Aim 1B. I then combined the saturated culture generation-10 and generation-250 yeast with the saturated culture reference yeast strain well-by-well in a 50:50 proportion. I plated the revived yeast mixtures in 96-well deep well microplates containing CM at a dilution of 1:1000 using a micropipette and a two-step dilution series. I incubated the competition assay microplates for 24 hours at 30°C. After 24 hours of growth, I collected samples for DNA

extraction and sequencing from each well and pooled them so that no evolutionary barcode overlap occurred within each pool. I transferred the 24-hour cultured yeast to fresh deep well microplates at a dilution of 1:1000 in CM, again using a 96-pin replicator, and incubated the newly inoculated microplates for an additional 24 hours. I repeated this transfer protocol one additional time, for a total of three periods of 24-hour incubation. Finally, I collected samples for DNA extraction and sequencing from each well of the 24-hour incubated microplates from the third incubation period. Here, I also pooled collected samples so that no evolutionary barcode overlap occurred within each pool.

Sample Processing. I processed the samples from the experimental evolution assays and sequencing-based competition assays in Aim 1B following the methods described in the "sample processing" section of Aim 1A. Here, however, I utilized a lyticase plus beads DNA isolation protocol to extract DNA for all yeast samples.

Sequence Data Processing. Ion Torrent sequencing recovered 125,158,974 library ion sphere particles. 33.6% of these reads were polyclonal, and 17.4% were low read quality, resulting in a final library of 62,541,139 high-quality reads (50% of total). I received sequence data in the form of fastQ files. I processed all fastQ files using R to create a read database containing 34,446,803 reads that perfectly matched our expected fragment sequences (forward primer, reverse primer, and yeast barcode sequence were all required to match our records exactly). I assigned the perfectly matched reads to experiments by forward and reverse barcode identity, and to yeast strains within each experiment by yeast barcode sequence. I used R to convert these reads to counts in order to determine the number of counts recovered for each yeast strain in each of the experiments. This process ultimately resulted in 24,652,722 counts that mapped to the correct yeast strains and experiment identities. In some instances, I detected yeast strains in experiments where they should not have been present; a total of 9,794,081 counts fall within this category. I used these counts to assess the distribution of contamination rates among experimental wells (Fig. 1.3). From these data, I produced a counts matrix (yeast strain by row, experiment identity by column) that I will use for all subsequent analyses. I detected unusually high contamination rates in some of the experiments included in this sequencing run (Fig. 1.3), I ultimately determined that these elevated contamination rates were due to primer contamination during primer assignment in the "Sample Processing" step of this sub-aim. I reprocessed these samples and submitted them for Ion Torrent sequencing a second time. This second Ion Torrent sequencing run recovered 138,151,588 library ion sphere particles. 49.2% of these reads were polyclonal, and 6.0% were low read quality, resulting in a final library of 64,554,726 high-quality reads (47% of total). After processing in R, my read database contained 40,465,262 reads that perfectly matched our expected fragment sequences. Once converted to counts, this second sequencing run resulted in 39,405,116 counts that mapped to the correct yeast strains and experiment identities. In this run, I also detected some yeast strains in experiments where they should not have been present; a total of 1,060,146 counts fall within this category. I used these counts to assess the distribution of contamination rates among experimental wells (Fig. 1.4) in the second sequencing run. I again produced a counts matrix from the counts data. Before conducting any statistical analyses, I will combine the matrices from the first sequencing run described in this section with those from the second, replacing highly contaminated experimental data from the first run with higher quality data from the second to produce a consensus matrix that minimizes contamination rate among experiments.

Future Work. The entirety of the wet-lab work for Aim 1B is complete. Sequence data processing is also complete, with the exception of combining the two counts datasets from the first and second sequencing runs. I have visually inspected the counts data from both sequencing runs, but I have not yet conducted rigorous statistical analyses or produced high-quality plots depicting evolutionary dynamics, changes in fitness, or fitness differences among treatments (but see Figure 1.2 for plot of preliminary fitness analyses). Preliminary review of the counts data from Aim 1B suggests that our sequencing-based competition assay can detect changes in yeast fitness (goal (4) for Aim 1) and indicates that ancestral yeast strains have equal fitness regardless of barcode identity (goal (5) for Aim 1). Rough plots of evolutionary dynamics and initial review of the degree of fitness changes across the evolutionary period suggest that haploid and diploid yeast, and yeast grown in different medias (CM, CM + ethanol, and CM + NaCl), differ in their evolutionary dynamics (goal (6) for Aim 1). Conducting the experimental evolution assays, competition assays, and sample processing for Aim 1B helped me to further refine experimental evolution system techniques and methodology (goal (1) for Aim 1). All experimental evolution, competition assay, and sample processing methods and techniques will follow strict, uniform, guidelines from this point forward.

Aim 1 Products. Aim 1 of my thesis has already produced research products that are in use throughout Aim 2; these products will be useful for all future research in the Botero Lab yeast experimental evolution system. Products include, 1) experimental evolution protocols for evolving yeast in liquid media, 2) sequencing-based competition assay protocols to describe yeast fitness and the change in yeast fitness through time, 3) standard sample processing protocols starting with yeast sample collection and culminating in library submission for sequencing, and 4) sequence data processing workflows to convert raw sequencing data to formatted counts matrices. Ultimately, I will also author a 'methods' publication describing our yeast experimental evolution system and the utility of the system for testing evolutionary theory through experimental evolution in barcoded yeast. This publication will likely draw on some data generated in Aim 2, as the data from Aim 1 were generated using heterogeneous, and, in some cases, non-ideal, methodology.

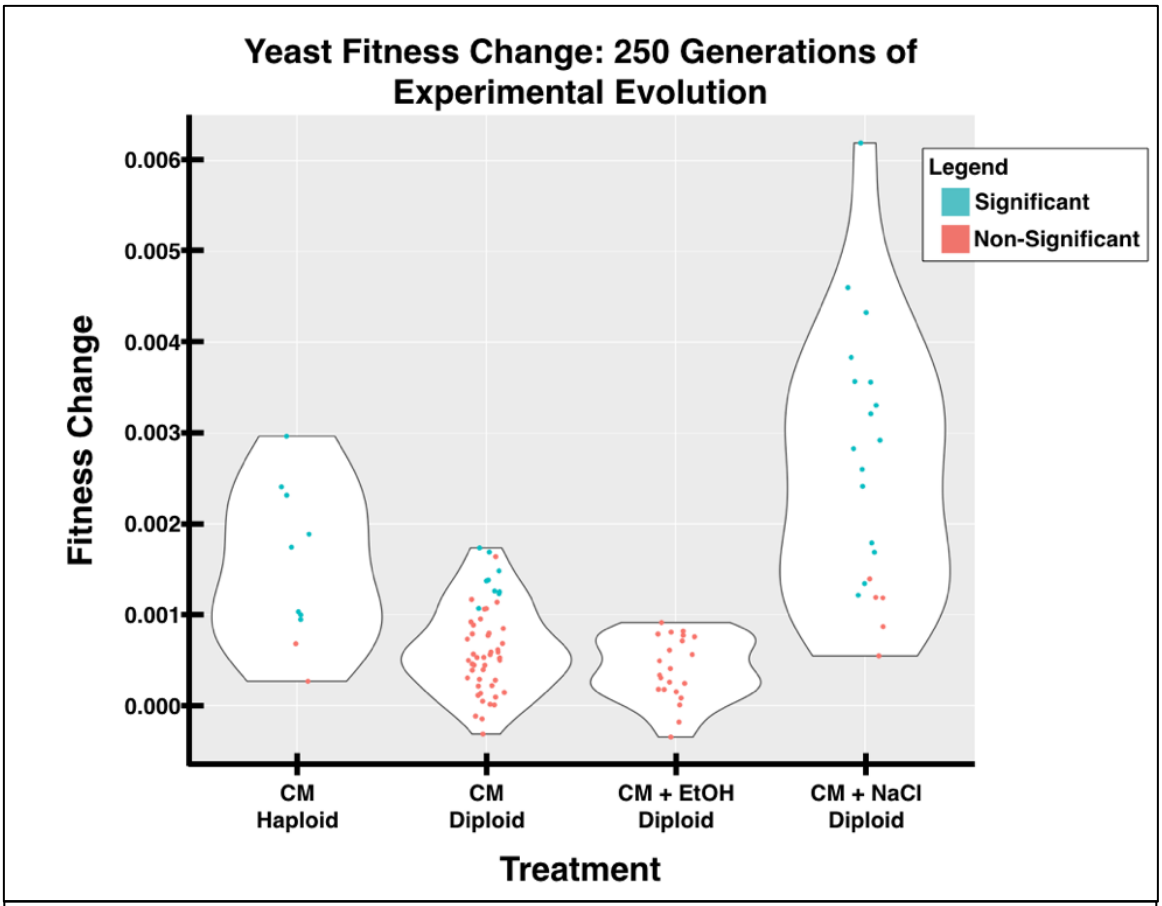


Figure 1.2. Preliminary fitness results from Aim 2B competition assays. violin-plot depicts fitness change for individual yeast strains over the 250-generation experimental evolution. Treatments depicted are: (1) complete minimal media, haploid yeast; (2) complete minimal media, diploid yeast; (3) complete minimal media plus ethanol, diploid yeast; and (4) complete minimal media plus sodium chloride, diploid yeast. Blue points depict significant changes in fitness between generation-0 and generation-250, Red points depict non-significant fitness changes. Figure produced in R (R Core Team, 2017).

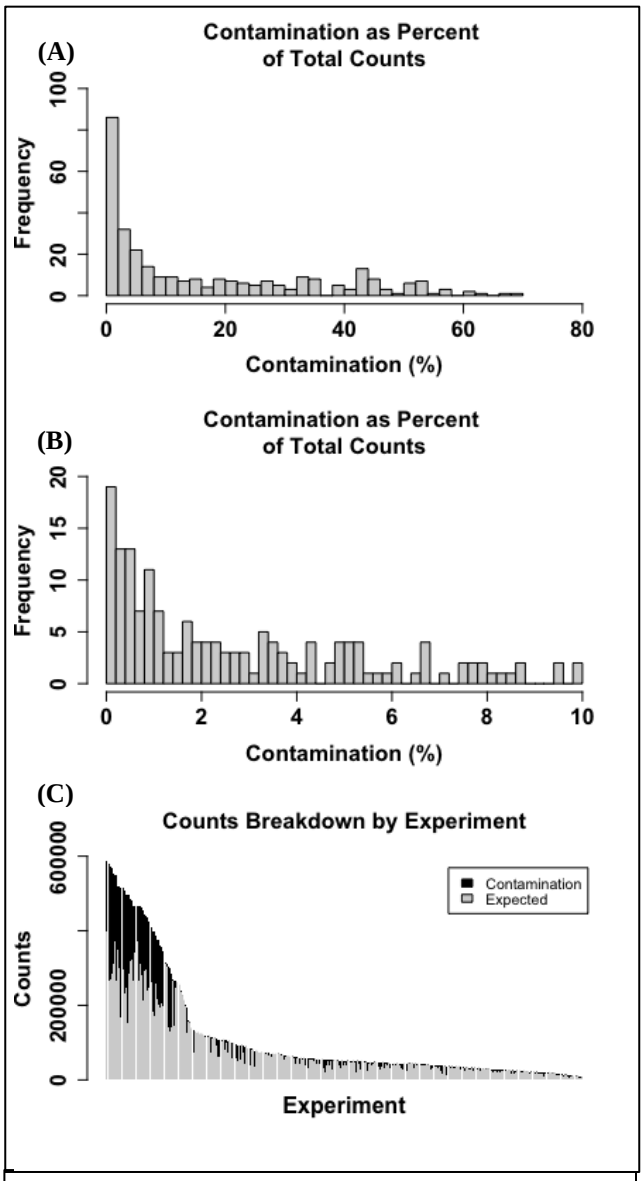


Figure 1.3. Contamination and counts summary information for Aim 1B sequence data processing, first sequencing run. (A) Percent contamination by experiment for all experiments in the sequencing run. (B) Percent contamination by experiment for all experiments with less than 10% contamination. (C) Total counts recovered for all experiments; bars are broken to show distribution of expected and contamination counts for each experiment. Figure produced in R (R Core Team, 2017).

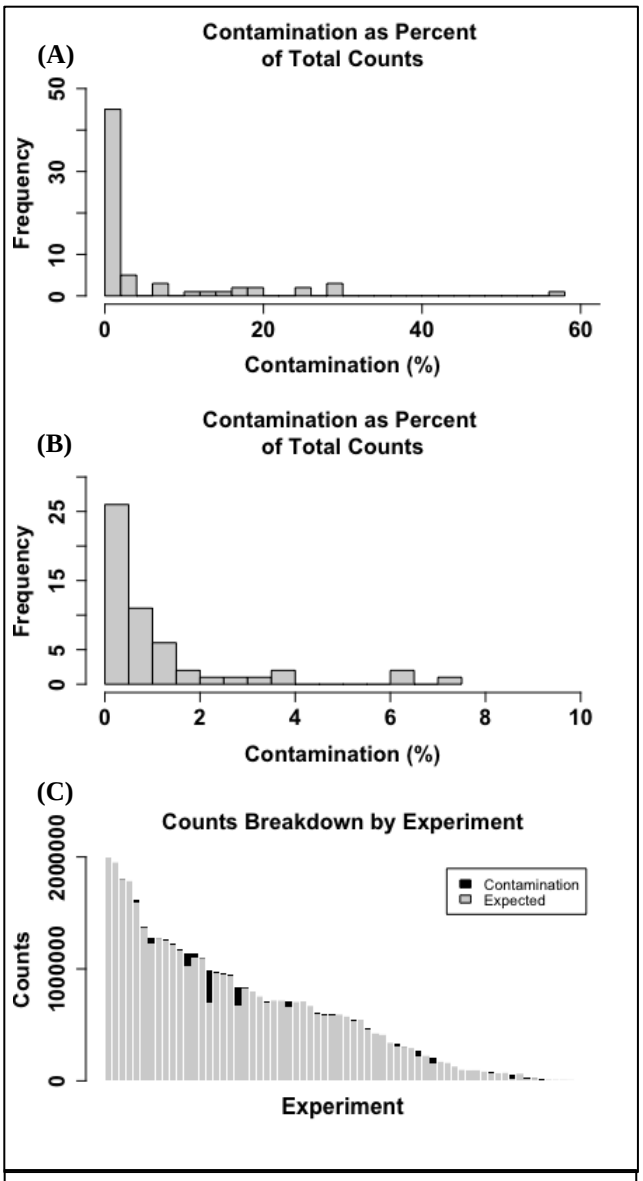


Figure 1.4. Contamination and counts summary information for Aim 1B sequence data processing, second sequencing run. (A) Percent contamination by experiment for all experiments in the sequencing run. (B) Percent contamination by experiment for all experiments with less than 10% contamination. (C) Total counts recovered for all experiments; bars are broken to show distribution of expected and contamination counts for each experiment. Figure produced in R (R Core Team, 2017).

Aim 2: Janzen's Physiological Barrier Hypothesis - Experimental Evolution

Summary. In this aim of my thesis, I will directly test Janzen's physiological barrier hypothesis (1967) through experimental evolution in yeast. Broadly, the hypothesis assumes that selective history shapes physiological tolerance such that populations adapt to the physiologically challenging conditions present in their environments. Specifically, it assumes that populations have physiological tolerances that match their selective history: populations from environments with uniform conditions have narrow physiological tolerances, while and those from fluctuating environments have broad physiological tolerances. Differences in physiological tolerance should translate to differences in realized barrier effectiveness: species with narrow physiological tolerance should be more restricted by physiological barriers than species with wide physiological tolerance when physiological barriers are of comparable magnitudes. To assess how selective history impacts stress tolerance and the effectiveness of physiological dispersal barriers, I first evolved yeast in physiologically challenging environments characterized by either uniform or fluctuating chemical stress concentration (Fig. 2.3 A. & B.). Yeast experienced one of six different uniform stress concentrations (0%, 20%, 40%, 60%, 80%, and 100% ancestral lethal limit) or one of five different fluctuating stress regimes (0%|40%, 0%|60%, 0%|80%, 20%|60%, and 40%|80% ancestral lethal limit) for the forty-nine-day experimental evolution period (~490 generations). Fluctuating stress treatments were alternated between the low- and high-stress concentrations each transfer, and all uniform and fluctuating selective treatments were replicated in three chemical stressors (NaCl, CuSO4, and Na2SO3). Using competition assays, I will assess how selective history shapes yeast survival and reproductive capacity in subsequent exposure to physiologically challenging conditions that can represent dispersal barriers. I define physiological barriers as environmental conditions that fall either above or below the environmental conditions experienced during the experimental evolution period for each selective history treatment (Figure 2.3 C. & D.). Here, I will compete evolved and ancestral yeast against a common ancestral strain in environments that differ in stressor concentration to quantify the change in survival and reproductive capacity for each evolved strain across several environmental contexts. I will interpret the results from these competition assays in the context of Janzen's physiological barrier hypothesis. I will determine if yeast from fluctuating selective histories have greater survival and reproductive capacities than yeast from uniform selective histories when exposed to physiological challenges that can act as dispersal barriers and/or if yeast from high-stress environments (regardless of fluctuation vs. uniform selective history) have greater survival and reproductive capacities than yeast from low-stress environments when exposed to physiological challenges. I will assess whether the results are general (similar for all three chemical stressors) or specific (different in at least one of the chemical stressors). The hypotheses that I test in Aim 2 compliment those tested in Aim 3: Aim 2 provides a test of the evolutionary mechanism underlying Janzen's physiological barrier predictions, while Aim 3 assesses whether Janzen's predictions are consistent with vertebrate diversification history and whether they can provide an explanation for diversity patterns. Aim 2 also uses experimental evolution to explore adaptation to uniform and fluctuating environmental conditions more generally. This, in itself, will improve our understanding of adaptation to constant and fluctuating environments as few experimental evolution studies have been conducted to explicitly assess these outcomes. To date, experimental evolution studies that have evaluated fluctuating versus constant selection tend to test only a few conditions, and these studies typically focus simply on comparing adaptation to one fluctuating selective regime to adaptation to one or two uniform selective regimes (e.g., Condon et al., 2013; Hughes et al., 2007; New et al., 2014; Ketola & Saarinen 2015; Ketola et al., 2013; Razinkov et al., 2014) or on the dynamics of adaptation to stochastically fluctuating environments (Karve et al., 2015a; Karve et al., 2015b; New et al., 2014). To my knowledge, no experimental evolution study has comprehensively evaluated adaptation to multiple uniform and fluctuating selective environments that differ in mean stress concentration, maximum stress concentration, and degree of fluctuation. Furthermore, the generality of these adaptive outcomes has not been tested across chemical stressors or by conducting competition assays across multiple environmental contexts.

Goals.

(1) Evaluate the evolutionary mechanism that underlies Janzen's physiological barrier hypothesis.

(2) Explore adaptation to fluctuating and uniform selective regimes more generally.

Research Questions.

(1) How does selective history in uniform versus fluctuating environmental conditions impact yeast survival and reproductive capacity in subsequent exposure to physiological challenges that can act as dispersal barriers?

(2) How do additional aspects of the selective regime (e.g., mean and maximum stress concentration) impact yeast survival and reproductive capacity in subsequent exposure to physiological challenges?

Hypotheses & Predictions.

(H1) Selection for flexibility facilitates response to novel stressful conditions, such that physiological barriers are smaller for organisms with fluctuating selective histories than for those with uniform selective histories.

(P1-1) Yeast from fluctuating selective histories will have greater survival and reproductive capacity than yeast from uniform selective histories when exposed to similar physiological challenges that can act as dispersal barriers (Fig. 2.1 A.).

(P1-2) Yeast from high-fluctuation selective histories will have greater survival and reproductive capacity than yeast from low-fluctuation selective histories when exposed to similar physiological challenges that can act as dispersal barriers (Fig. 2.1 B.).

(P1-3) Yeast from high-stress fluctuating selective histories will have similar survival and reproductive capacity to yeast from low-stress fluctuating selective histories when exposed to similar physiological challenges that can act as dispersal barriers (Fig. 2.1 C.).

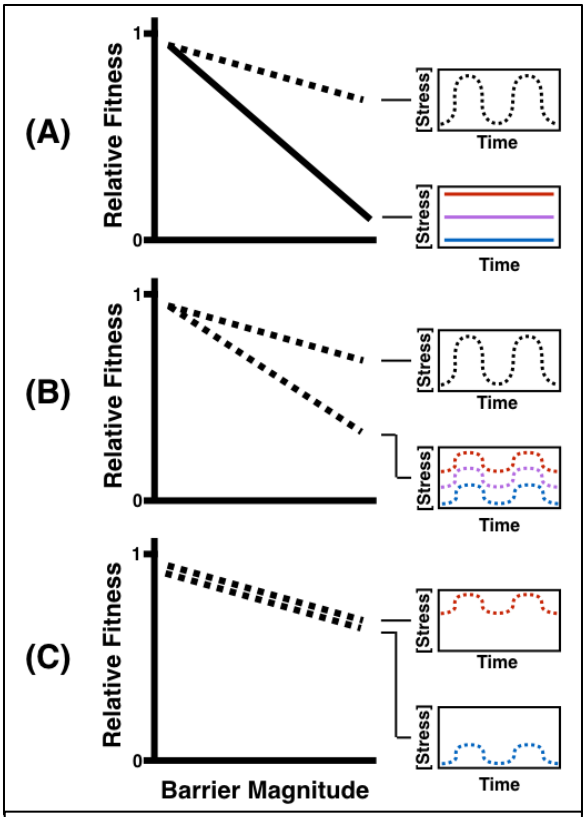


Figure 2.1. Conceptual illustration depicting predictions for H1. (A) Prediction (P1-1). (B) Prediction (P1-2). (C) Prediction (P1-3). Dashed lines are fluctuating selective histories, solid lines are uniform selective histories. Insets show selective history regime for lines on main panels.

(H2) Amount of exposure to stress facilitates response to novel stressful conditions, such that physiological barriers are smaller for organisms with selective histories in stressful environments than benign environments.

(P2-1) Yeast from high stress concentration selective histories (measured as the maximum stress level in selective history) will have greater survival and reproductive capacity than yeast from low stress concentration selective histories when exposed to physiological challenges that can act as dispersal barriers, regardless of the degree of fluctuation in selective history stress regime (Fig. 2.2 A.).

(P2-2) Yeast from high-stress concentration selective will have greater survival and reproductive capacity than yeast from low-stress concentration selective histories when exposed to physiological challenges that can act as dispersal barriers; yeast with uniform selective histories will have greater survival and reproductive capacity than yeast from fluctuating selective histories that share the same maximum stress concentration (Fig. 2.2 B.).

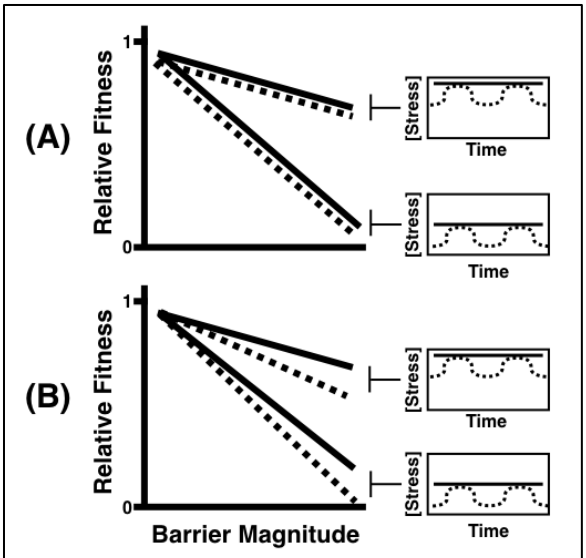


Figure 2.2. Conceptual illustration depicting predictions for H2. (A) Prediction (P2-1). (B) Prediction (P2-2). Dashed lines are fluctuating selective histories, solid lines are uniform selective histories. Insets show selective history regime for lines on main panels.

Aim 2A: Evolve yeast in selective environments characterized by uniform and fluctuating chemical stressor concentration. To address the goal, research questions, and assumptions of this aim, I first estimated the minimum stressor concentration that resulted in no growth for my unevolved yeast strains (i.e., the ancestral lethal limit). I then generated yeast with varied selective histories through ~500 generations of experimental evolution. I evolved yeast in one of eleven environmental regimes across three chemical stressors (n=33 total treatments). Of the eleven regimes, six were characterized by the same stress concentration each day (Uniform: 0%, 20%, 40%, 60%, 80%, and 100% ancestral lethal limit) and five were characterized by daily alternating stress concentrations (Fluctuating: 0%|40%, 0%|60%, 0%|80%, 20%|60%, and 40%|80% ancestral lethal limit) (Fig. 2.3 A & B). At the end of the experimental evolution period, I sequenced samples of all yeast populations from generations 10, 70, 130, 190, 250, 310, 370, 430, and 490 to assess changes in population composition through time and to inform competition assay planning for Aim 2B (Sequencing results; Fig. 2.5).

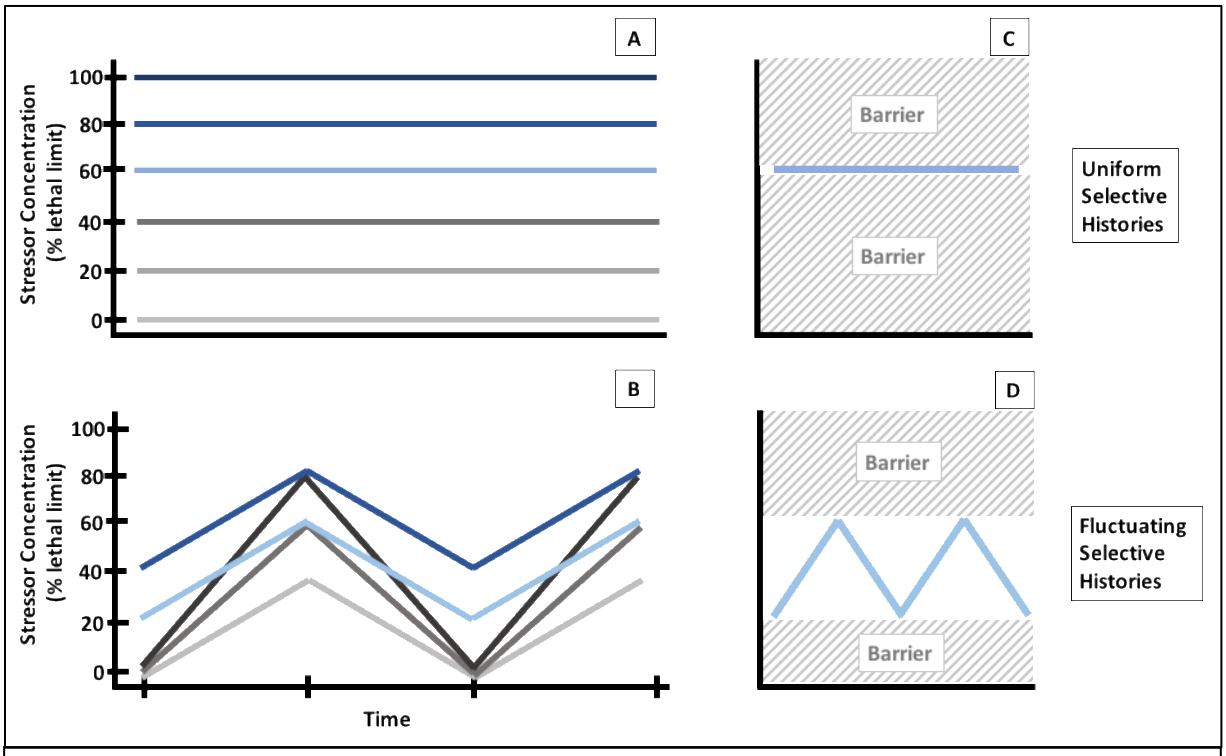


Figure 2.3. Conceptual illustrations depicting the eleven selective histories for the experimental evolution experiment. (A) Stress regimes for the six uniform selective histories. (B) Stress regimes for the five fluctuating selective histories. (C) Illustration of what constitutes a physiological barrier for uniform selective history treatments. (D) Illustration of what constitutes a physiological barrier for fluctuating selective history treatments.

Ancestral Lethal Limit. Prior to carrying out the experimental evolution project, it was first necessary to determine the lethal limit of the ancestral (unevolved) yeast. I did this by growing ancestral yeast in complete minimal media (CM) spiked with NaCl, CuSO4, or Na2SO3 + Tartaric Acid. I revived ancestral yeast from freezer stocks, diluted them 1:1000 in liquid CM on 96-well deep well microplates and incubated the microplates for 24 hours at 30°C. I then replicate plated these 24-hour cultures to new deep well microplates, filled with fresh media, at a dilution of 1:1000. The first replicate microplate contained CM + NaCl at several concentrations, the second contained CM + CuSO4, and the third contained CM + (Na2SO3+Tartaric Acid). I incubated the microplates for 24 hours at 30°C. After 24 hours, I removed the deep well microplates from the incubator, resuspended the yeast in each well, removed 100ul aliquots from a subset of the wells, and plated these aliquots on a 96-well shallow well microplate for spectrophotometric reading. I measured the optical density at 600nm for all 24-hour yeast samples to compare growth in stress media to growth to control conditions (CM with no chemical stress). I returned the deep well microplate to the incubator, and collected aliquots for spectrophotometric reading from the rest of the wells after an additional 24 hours of incubation (total growth = 48 hours). I measured the optical density for the 48-hour yeast samples as specified above. I ran these trials several times to ensure that the results were accurate and repeatable. I

defined the yeast ancestral lethal as the chemical concentration in which no growth occurred over a 48-hour incubation period. The ancestral lethal limit for NaCl is 0.342 molar, the lethal limit for CuSO4 is 8.0 micromolar, and the lethal limit for Na2SO3+Tartaric Acid is 0.8 millimolar (Fig. 2.4).

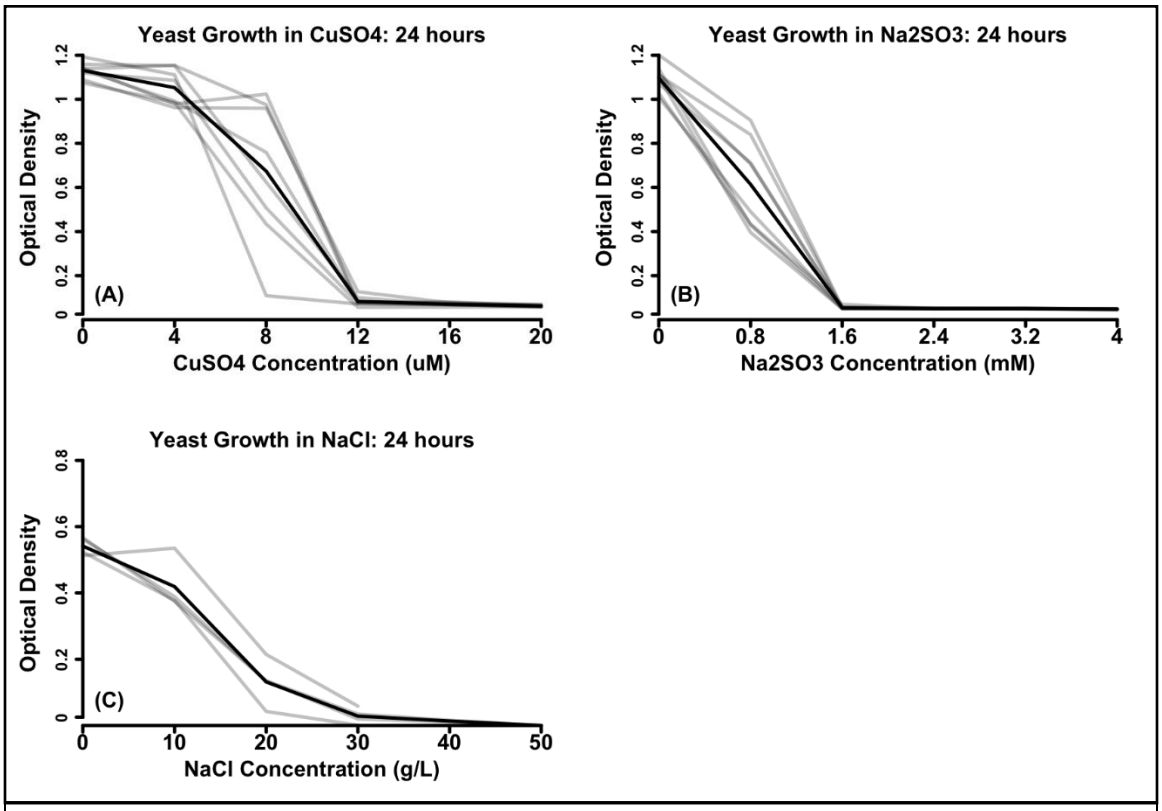


Figure 2.4. Preliminary lethal limit data. (A) Yeast growth after 24-hours in copper sulfate. (B) Yeast growth after 24-hours in sulfite plus tartaric acid. (C) Yeast growth after 24-hours in sodium chloride. Light grey lines depict individual yeast strains replicated across each stress concentration treatment. Black lines depict averages. Data for (A) and (B) are from a different date than (C). Figure produced in R (R Core Team, 2017).

Experimental Evolution. To address the goals of this aim, I revived diploid yeast from freezer stock, combined them in pairs to form mixed genotype populations, and evolved the populations in liquid media for 49 transfers (490 generations) (See Table 2.1 for experimental design). I chose to use diploid, rather than haploid yeast, because yeast are known to "diploidize" during experimental evolution studies (Gerstein et al., 2006; Gerstein et al., 2011) For the experimental evolution assay, I replicate plated ancestral yeast onto six 96-well deep well microplates (2x CM + NaCl, 2x CM + CuSO4, 2x CM + Na2SO3 & Tartaric Acid) (Table 2.1 B.). Each of the six microplates contained eleven experimental evolution treatments, with each column corresponding to a different treatment. Column treatments, as the percentage of ancestral lethal limit, were the same for all plates and only differed by chemical id. The first six columns in each plate were evolved under uniform stress regimes (no daily fluctuation) at 0%, 20%, 40%, 60%, 80%, and 100% of the ancestral lethal limit, respectively. The next five columns in each plate were evolved under daily fluctuating stress regimes (alternate stress concentration each transfer) at 0%|40%, 0%|60%, 0%|80%, 20%|60%, and 40%|80% of the ancestral lethal limit, respectively. All wells in each column of each experimental evolution microplate represent biological replicates that differ only in genotype (strain barcode identity), but not in initial fitness. Wells in the same position (e.g., A1) on different microplates that share the same chemical stressor identity represent direct biological replicates (same genotype). I collected Yeast samples for DNA extraction, processing, and DNA sequencing from all wells in all plates after the first transfer (generation10), after the 49th transfer (generation 490), and every six transfers in between (generations 70, 130, 190, 250, 310, 370, and 430) (Table 2.1 C.).

(A)	# experimental wells	strains/ experiment	Ploidy	Dilution	Selective Environment	Stress concentration 1	Stress Concentration 2
Treatment 1	16	2	Diploid	1:1000	Uniform	0%	NA
Treatment 2	16	2	Diploid	1:1000	Uniform	20%	NA
Treatment 3	16	2	Diploid	1:1000	Uniform	40%	NA
Treatment 4	16	2	Diploid	1:1000	Uniform	60%	NA
Treatment 5	16	2	Diploid	1:1000	Uniform	80%	NA
Treatment 6	16	2	Diploid	1:1000	Fluctuating	100%	NA
Treatment 7	16	2	Diploid	1:1000	Fluctuating	0%	40%
Treatment 8	16	2	Diploid	1:1000	Fluctuating	0%	60%
Treatment 9	16	2	Diploid	1:1000	Fluctuating	0%	80%
Treatment 10	16	2	Diploid	1:1000	Fluctuating	20%	60%
Treatment 11	16	2	Diploid	1:1000	Fluctuating	40%	80%

(B)	Chemical Stressor	Plate ID
Plate 1	NaCl	SALT A
Plate 2	NaCl	SALT B
Plate 3	CuSO4	COPR A
Plate 4	CuSO4	COPR B
Plate 5	Na2SO3 + Tartaric Acid	SULF A
Plate 6	Na2SO3 + Tartaric Acid	SULF B

(C)	Transfer
Evolution DNA Samples	1, 6, 13, 19, 25, 31, 37, 43, 49
Competition Assay Material	1, 49
Stock Plates	1, 49 *plus 1/ week between transfers 1 and 49

Table 2.1. Experimental Design for Aim 2. (a) Treatment information for experimental evolution assay plates; number of experimental wells per treatment, number of strains per experimental well, yeast ploidy, inoculation dilution, selective environment, and stress concentrations. (b) Plate information for experimental evolution assay; two plates per chemical stressor plus information on plate ID. (c) Collection time points for experimental evolution DNA samples and -80°C stock plates as well as starting material for competition assays.

Revive Yeast. I revived yeast from single-colony derived stock cultures stored at -80°C in 96-well shallow well microplates. Each well in the stock microplates contained 100ul saturated yeast culture plus 100ul 30% glycerol in H_2O. To revive the yeast, I thawed the stock microplates and then thoroughly resuspended the yeast in each well using a micropipette. I removed ~0.6ul of thawed culture stock from each well using a pin-replicator tool and transferred the full volume to the corresponding well (same plate position) of a 96-well deep well microplate containing 600ul of complete minimal media (CM) per well (~1:1000 dilution). I then covered the inoculated deep well microplate with a breathable membrane and incubated it for 24 hours at 30° Celsius. Following incubation, I transferred the yeast to a new deep well microplate, containing 600ul of fresh CM per well, using the same pin-replicator tool. I incubated the newly inoculated microplates for 24 hours at 30° Celsius. I used the 24-hour yeast cultures from this second transfer as the starting material for the experimental evolution trials.

Evolution in Liquid Media. I mixed 176 revived yeast strains to form 88 unique pairs with approximate starting ratios of 50:50 (1^{st} strain in pair: 2^{nd} strain in pair). After mixing, I arrayed the 88 pairs across the first eleven columns of a 96-well deep well microplate and incubated them for 24 hours at 30°C. At 24 hours, I resuspended the yeast, collected 60ul samples from each well, pooled all 60ul samples to form one consolidated sample, and saved the sample at -80°C for future DNA extraction and sequencing. From the same plate, I also removed 100ul aliquots from each well (no pooling), mixed them 50:50 with a 30% glycerol solution, and stored them in a 96-well shallow well microplate at -80°C; this plate serves as the ancestral stock plate for the experiment, it contains generation 10, day 1, yeast. I then prepared six deep well microplates containing complete minimal media (CM) plus either NaCl, CuSO4, or Na2SO3 & Tartaric Acid according to the experimental design (Table 2.1). After adding 600ul of the appropriate media to each well of the six microplates, I replicate plated the 24-hour cultured yeast to each plate using a 96-pin-replicator tool at a dilution of ~1:1000. I then incubated the microplates for 24 hours at 30°C. After 24 hours, I prepared six new microplates (now using the second stress level listed for the fluctuating treatments in table 2.1 A.), removed the six 24-hour culture microplates from the incubator, and transferred the yeast, using the pin replicator tool, from each of the six 24-hour culture microplates to the corresponding microplate in the newly created set. I repeated this process every 24 hours until the yeast in each plate had experienced 49 transfer events (~490 generations). Every six transfers, I collected samples for DNA extraction and sequencing from each well in

each microplate. I pooled these samples by microplate and stored the pooled samples at -80°C until the end of the experimental evolution period. Once each week, I removed 100ul from each well of each microplate (no pooling), mixed the samples 50:50 with a 30% glycerol solution, and stored them in a 96-well shallow well stock plate at -80°C. These stock plates served as backup copies in case I needed to restart the evolution from any time point, and will also serve as starting material for future competition assays.

Sample Processing. I extracted DNA from each of the pooled samples collected at generations 10, 60, 130, 190, 250, 310, 370, 430, and 490 using the single-tube version of the Zymo Scientific Fungal/Bacterial DNA Miniprep kit. Additionally, I extracted nine samples from individual wells on the generation-490 microplates as 'single-well controls.' These controls will allow me to assess contamination rates; here, contamination refers to yeast that were unintentionally spread to the incorrect experimental evolution wells over the course of the 50-day evolutionary period. I also ran three blanks, containing just Millipore water, through the DNA extraction protocol as an additional control. I quantified DNA concentration prior to downstream processing using two complimentary methods. First, I measured DNA concentration using a Qubit High Sensitivity DNA Quantification Kit and a Qubit 3.0 Flourometer. I then confirmed the measured concentrations by running a 1% Agarose gel and comparing sample band intensity to Flourometer concentration data. I subsequently attached a unique combination of forward and reverse Ion Torrent sequencing primers to DNA fragments in each sample using a thermocycler (94°C for 5 minutes; 25 cycles - 94°C for 10 seconds, 50°C for 10 seconds, 72°C for 10 seconds; 72°C for 5 minutes). I ran three 'primer addition blanks', containing Millipore water in place of isolated yeast DNA, through the thermocycler alongside the sample reactions as a further control. I measured PCR product DNA concentrations using the Qubit Flourometer (described above) and confirmed the measurements using a 1% Agarose gel. I visually inspected Agarose gel bands to ensure that each sample produced a single clean band at the desired fragment length. I then pooled All PCR products to form a single library. I performed a gel extraction on the library using a Zymoclean Gel DNA Recovery Kit as a final purification step. I used a low-cut excised gel fragment, from the region just below the main band on the 1% Agarose gel, as the sample input for the gel extraction. I quantified the DNA concentration of the pooled, purified library a third time using the Qubit Flourometer (described above). Finally, I delivered the purified library to the Dorsett Lab at Saint Louis University for sequencing. There, DNA concentration and fragment size were quantified using a GC Trace (Agilent DNA 1000) prior to Ion Torrent sequencing. Ion Torrent sequencing was performed according to standard protocols.

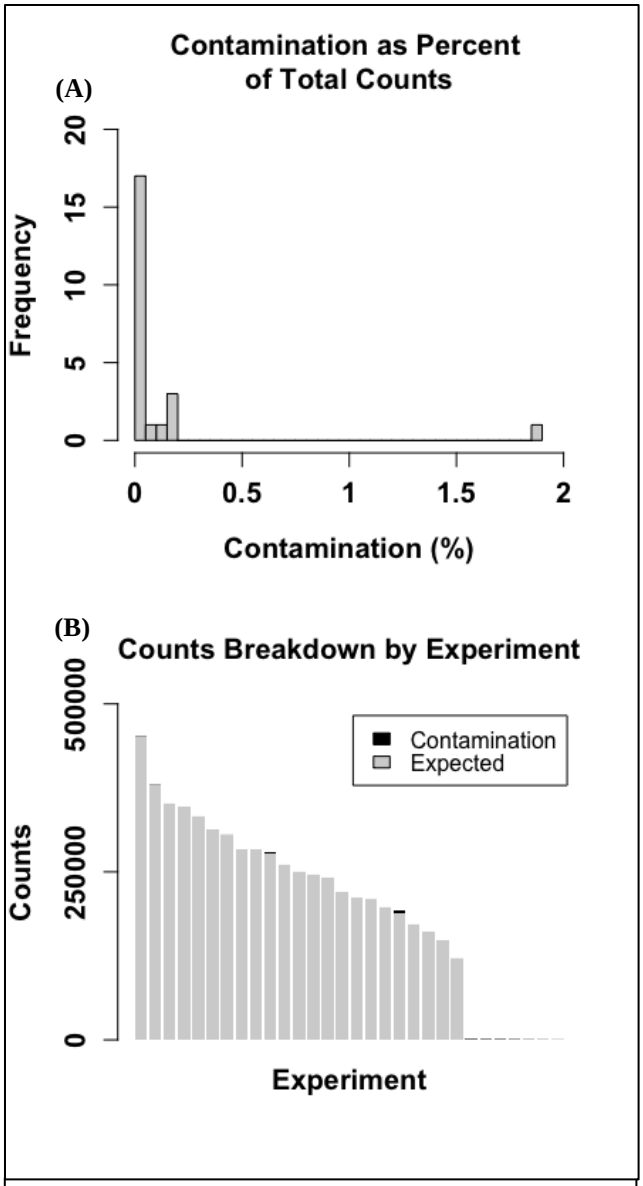


Figure 2.5. Contamination and counts summary for Aim 2A. sequence data. (a) Contamination as percent of total counts for all single well controls. (b) Total counts recovered for single well controls, DNA blanks, and PCR blanks; bars are broken to show distribution of expected and contamination counts for each control. The final seven columns in (b) correspond to the three DNA extraction blanks, three PCR blanks, and one single-well control that went extinct over the course of the experimental evolution period. Figure produced in R (R Core Team, 2017).

Sequence Data Processing. Ion Torrent sequencing recovered 134,347,324 library ion sphere particles. 40.2% of these reads were polyconal, and 7.1% were low read quality, resulting in a final library of 73,233,378 usable reads (55% of total). I received sequence data in the form of fastQ files. I processed all fastQ files using R to create a read database containing 51,798,140 reads that perfectly matched our expected fragment sequences (forward primer, reverse primer, and yeast barcode sequence were all required to match our records exactly). I assigned the perfectly matched reads to experiments by forward and reverse barcode identity, and to yeast strains within each experiment by yeast barcode sequence. I used R to convert these assigned reads to counts in order to determine the number of counts recovered for each yeast strain in each of the experiments. This process ultimately resulted in 51,786,243 counts that mapped to the correct yeast strains and experiment identities. The experimental design for this sequencing run was slightly different than most previous runs; in this case, all wells from each experimental evolution microplate were pooled to form single, pooled samples prior to DNA extraction at each collection point. This pooling method makes it impossible to detect contamination rates for individual experiments, and requires that single-well controls, DNA extraction blanks, and PCR blanks are used to estimate contamination percentages. Among these controls and blanks I detected a total of 11,897 contamination counts (Fig. 2.5). From the sequencing data, I produced a counts matrix (yeast strain by row, experiment identity by column) that I will use in all subsequent analyses.

Future Work. The majority of the work for Aim 2A is complete. I still need to produce plots and summary figures depicting the change in barcode proportion through time and highlighting any differences in evolutionary dynamics among treatments and/ or chemical stressors. Visual inspection of these plots, possibly accompanied by statistical analysis, will help to inform/ refine the competition assay experimental design described in Aim 2B. I must also conduct analyses of contamination rates from the experimental evolution samples; these contamination rates set the maximum resolution for detection of fitness changes in subsequent experimental work using these yeast strains.

Aim 2B: Phenotyping ancestral and evolved yeast. To address the goal, research questions, and assumptions of Aim 2, I will use competition assays to understand how selective history impacts yeast fitness in subsequent exposure to physiological challenges. In these assays, I will compete evolved (generation-490) and ancestral (generation-10) yeast against a common reference strain in a range of environments that vary in chemical stress concentration. I will measure the relative fitness of the ancestral (generation-10) and evolved (generation-490) yeast in all conditions and I will quantify the change in fitness for each yeast strain over the 480 generations of experimental evolution. I will interpret the results of these competition assays in the context of the physiological barrier hypothesis.

Sequencing-based competition assay. I will phenotype ancestral (generation-10) and evolved (generation-490) yeast using a sequencing-based competition assay. In this assay, I will compete evolved and ancestral yeast against a single reference strain for three transfers (30 generations). These competition assays will allow me to identify fitness differences among evolved strains and allow me to calculate the change in fitness for each strain over the 480-generation experimental evolution period. Replicate competitions will be carried out across three media conditions, CM (no stress), CM + 40% ancestral lethal limit (medium stress), and CM + 80% ancestral lethal limit (high stress), for each of the three chemical stressors (NaCl, CuSO4, Na2SO3 & Tartaric Acid). In these competition assays, I will only challenge yeast with the chemical stressor that they were exposed to during the evolutionary period (e.g., NaCl evolved yeast competed in multiple NaCl concentrations, but not in any CuSO4 or Na2SO3 environments). Time and resource limitations require that I restrict the number of samples that I run through the competition assay to a subset of the eleven selective regimes. I have not yet decided exactly which of the treatments to include in these competition assays; tentatively, I plan to include three uniform selective history treatments and three fluctuating selective history treatments. Good candidates will differ in their selective regime means, minimums, and maximums, and will have selective histories in environments with stress concentrations that fall below, encompass, and fall above the stress concentrations used as challenges in this set of competition assays. Regardless of these decisions, the general form of the sequencing-based competition assay will be as follows. I will revive generation-10 and generation-490 yeast according to the protocol outlined in the "revive yeast" section of Aim 2A. After the yeast are revived, I will fill each well of a 96-well deep well microplate with 600ul of media according to the competition assay design. I will then transfer the revived yeast to the appropriate wells on the microplate at a dilution of ~1:1000. I will add the same reference strain to each well of the newly inoculated plate for a 50:50 proportion of reference strain yeast to experimental evolution yeast. I will incubate the competition assay microplate for 24 hours at 30°C. At 24 hours, I will resuspend the yeast and collect samples for DNA extraction and sequencing from each well. These samples will be used to determine the initial proportions of reference vs. experimental evolution yeast in

each well. This step is necessary because the reference and experimental evolution yeast strains will not start in exactly equal proportions; exact knowledge of starting proportions is necessary for fitness calculations. After sample collection, I will transfer the 24-hour cultured yeast to a fresh microplate at a dilution of ~1:1000, keeping treatment conditions the same as the first day. I will incubate the newly inoculated plate for an additional 24 hours. After this second 24-hour incubation, I will transfer the yeast to fresh media in a new microplate one final time following the exact protocol used in the previous transfer. After incubating the new plate for an additional 24 hours, I will again collect samples for DNA extraction and sequencing from each well. The final products of the competition assay will include four sets of DNA samples for sequencing: (1) Ancestral yeast from the first day of the competition assay (measure: initial proportion ancestral yeast), (2) ancestral yeast from the final day of the competition assay (measure: final proportion ancestral yeast), (3) Evolved yeast from the first day of the competition assay (measure: initial proportion evolved yeast), and (4) evolved yeast from the final day of the competition assay (measure: final proportion evolved yeast).

Sample Processing. I will extract DNA from all samples collected during the competition assay in Aim 2B using the single-tube version of the Zymo Scientific Fungal/Bacterial DNA Miniprep kit. Because all samples will contain the same reference yeast strain, no additional sample pooling can be done during the DNA extraction step. I will include blanks and single well controls at appropriate points throughout sample processing as described in the "sample processing" section of Aim 2A. As described in Aim 2A, I will quantify DNA concentration after DNA extraction and prior to downstream processing using a Qubit High Sensitivity DNA Quantification Kit. I will confirm these measurements by running a 1% Agarose gel. I will then attach a unique combination of forward and reverse Ion Torrent sequencing primers to each DNA sample using a thermocycler (94°C for 5 minutes; 25 cycles - 94°C for 10 seconds, 50°C for 10 seconds, 72°C for 10 seconds; 72°C for 5 minutes). I will quantify PCR product DNA concentrations using the Qubit Flourometer and confirm the results using a 1% Agarose gel. Additionally, I will visually inspect the bands on the Agarose gel to ensure that each sample produces a single clean band at the desired fragment length. I will pool all PCR products according to their concentrations to form a single library. Because the gel extraction (Zymoclean Gel DNA Recovery Kit) purification method produced clean results in Aim 2A, I will repeat the method here using the same protocol. I will quantify the DNA concentration of the pooled, purified library using the Qubit Flourometer before sending the sample for sequencing. Finally, I will deliver the purified library to the Dorsett Lab at Saint Louis University for Ion Torrent sequencing. There, DNA concentration and fragment size will be quantified using a GC Trace (Agilent DNA 1000) prior to Ion Torrent sequencing. Ion Torrent sequencing will be performed according to the same specifications used in all previous sequencing runs.

Sequence Data Processing. I will carry out sequence data processing for the experiments included in Aim 2B exactly as specified in the "Sequence Data Processing" section of Aim 2A.

Future Work. I will interpret sequencing results in the context of the physiological barrier hypothesis. I will first look for evidence of adaptation to the environmental conditions in all treatments, this will manifest as an increase in fitness from generation-10 (ancestral) to generation-490 (evolved) for individual yeast strains. I will then determine if there are differences in fitness among yeast strain from different selective histories in the various competition assay challenge conditions. Any differences, or lack thereof, will be discussed in terms of the support of lack of support that they lend to the evolutionary assumptions of the physiological barrier hypothesis.

Aim 2 Products. Aim 2A of my thesis has already produced research products that will be utilized in Aim 2B as well as in future experimental work in the Botero Lab yeast experimental evolution system. The yeast evolved in different selective histories for Aim 2A will be available for any future research projects in the Botero lab. I will use these yeast to carry out Aim 2B of my proposal, and the -80°C freezer stocks of these yeast will be saved for future undergraduate and graduate projects requiring yeast with different selective histories or prior exposure to selection in the presence of chemical stressors. Ultimately, I will publish the findings from Aim 2 of my thesis proposal in a manuscript evaluating the evolutionary assumptions of Janzen's physiological barrier hypothesis.

Aim 3: Janzen's Physiological Barrier Hypothesis – Biogeographical Study

Summary. Janzen's physiological barrier hypothesis argues that the magnitude of physiological barriers mediates the dispersal of organisms (i.e., gene flow) among suitable environments. Within this framework, the magnitudes of thermal barriers are determined by the interaction between temperature seasonality and the breadth of a species thermal niche. Thermal barriers to dispersal are therefore predicted to be higher in the low-temperature seasonality tropics, where species have narrow thermal niches and thermal regimes overlap little across elevations. In contrast, in the high-temperature seasonality temperate zone, species have broader thermal niches and thermal regimes overlap markedly across elevations. Although Janzen's original intent was not an attempt to explain latitudinal gradients of diversity, the physiological barrier hypothesis has since been suggested as plausible explanation for the higher species richness observed in the tropics (Ghalambor et al., 2006). This particular view rests on two major assumptions: (1) temperature seasonality varies geographically and is positively correlated with latitude; (2) thermal niche breadth evolves to match the temperature regime in a species' selective environment, such that thermal niche breadth is also positively correlated with latitude. If these assumptions are true, Janzen's hypothesis predicts that thermal barrier height varies geographically and is negatively correlated with latitude, temperature seasonality, and species thermal niche breadth, leading to the observed local, regional, and global patterns of biodiversity.

In Aim 3, I propose an evaluation of Janzen's central prediction that physiological barriers to dispersal are more effective in the tropics (Aim 3: H1). I also propose an assessment of the extent to which variation in the magnitude of physiological barriers to dispersal can provide an explanation for global patterns of montane species richness (Aim 3: H2). Given that Janzen's ideas were specifically developed with elevational gradients of temperature in mind, I will describe this aim using these parameters. However, my plan is to extend a similar framework to other environmental gradients that may serve as barriers to dispersal in order to assess the generality of Janzen's physiological barrier mechanism and its potential overall contribution to the latitudinal diversity gradient. To test the hypothesis that physiological barriers to dispersal are higher in more tropical areas, I will quantify the magnitude of thermal barriers that bisect the distributions of allopatrically distributed sister species (Fig 3.1 A.; Avian sister species). I will do this using the path of least resistance between all border cells of sister species ranges using river flow models (Fig 3.1 B.). I predict that the thermal barriers that separate sister species in tropical mountains are smaller than the thermal barriers that separate sister species in temperate mountains (Fig 3.1 C.). This prediction stems from the idea that smaller thermal barriers are necessary to drive allopatric speciation in tropical lineages, hence the statement that "mountain passes are higher in the tropics" (Janzen, 1967). Next, I will determine if variation in thermal barrier magnitude translates to variation in montane species richness. Here, I predict that species richness is greater in montane regions with large thermal barriers to dispersal (i.e., where thermal barriers of small magnitudes can drive allopatric speciation) than in montane regions with small thermal barriers to dispersal. I will test these two hypotheses using avian, amphibian, and mammalian sister species pairs globally and, in all analyses, I will consider several biological and environmental covariates that may shape patterns of thermal barrier magnitude and species richness (Aim 3: secondary questions).

Goals.

1. Test the core prediction of the physiological barrier hypothesis that thermal barriers to dispersal actually decrease in magnitude as we move from the tropics to the temperate zone.

2. Assess the extent to which the physiological barrier hypothesis can explain global patterns of extant species richness.

Main Questions.

1. Are physiological barriers to dispersal higher in more tropical areas?

2. Is species richness greater in montane regions with larger thermal barriers to dispersal than in montane regions with smaller thermal barriers to dispersal?

Hypotheses & Predictions.

(H1) Physiological barriers to dispersal are higher in more tropical areas.

(P1) The thermal barriers that separate sister species in tropical mountains are smaller than the thermal barriers that separate sister species in temperate mountains.

(H2) Physiological barrier magnitude governs montane species richness patterns.

(P2) Species richness is greater in montane regions with large thermal barriers to dispersal than in montane regions with small thermal barriers to dispersal

Secondary Questions.

1. How does proximity to large water bodies effect thermal barrier magnitude?
   Expectation: Proximity to large bodies of water reduces temperature seasonality. Sister species will be separated by smaller thermal barriers near large bodies of water.

2. How does mountain mass effect thermal barrier magnitude?
   Expectation: High mountain mass reduces temperature seasonality. Sister species will be separated by smaller thermal barriers in high-mountain mass montane areas.

3. How does diurnal temperature fluctuation effect thermal barrier magnitude?
   Expectation: Diurnal temperature fluctuation increases temperature variability, even in areas of low-temperature seasonality. Sister species will be separated by larger thermal barriers in areas with pronounced diurnal temperature fluctuation.

4. How do extreme/ rare climate events effect thermal barrier magnitude?
   Expectation: extreme/ rare climate events can increase temperature regime variability, even in areas of low-temperature seasonality. Sister species will be separated by larger thermal barriers in areas with less frequent and/ or less severe extreme/ rare climate events.

5. How does thermal barrier magnitude vary with elevation? Are the magnitudes of thermal barriers separating high elevation sites similar to the magnitudes of thermal barriers separating low elevation sites?
   Expectation: Temperature regimes can be more variable at high elevations than low elevations. Low-elevation sister species will be separated by larger thermal barriers than high-elevation sister species.

6. How does thermal barrier magnitude differ across vertebrate (mammal, bird, amphibian) clades?
   Expectation: Mammals, birds, and amphibians have different physiologies and life histories. Amphibian sister species will be separated by the smallest thermal barriers, mammals by intermediate size thermal barriers, and birds by the largest thermal barriers.

7. How do life history strategies that allow species to avoid harsh environmental conditions (e.g., through seasonal migration or hibernation) effect thermal barrier magnitude?
   Expectation: life history strategies that allow species to avoid harsh environmental conditions reduce the range of temperatures that species are exposed to. Sister species that avoid harsh environmental conditions will be separated by smaller thermal barriers than sister species that are exposed to the full range of seasonal temperature variation.

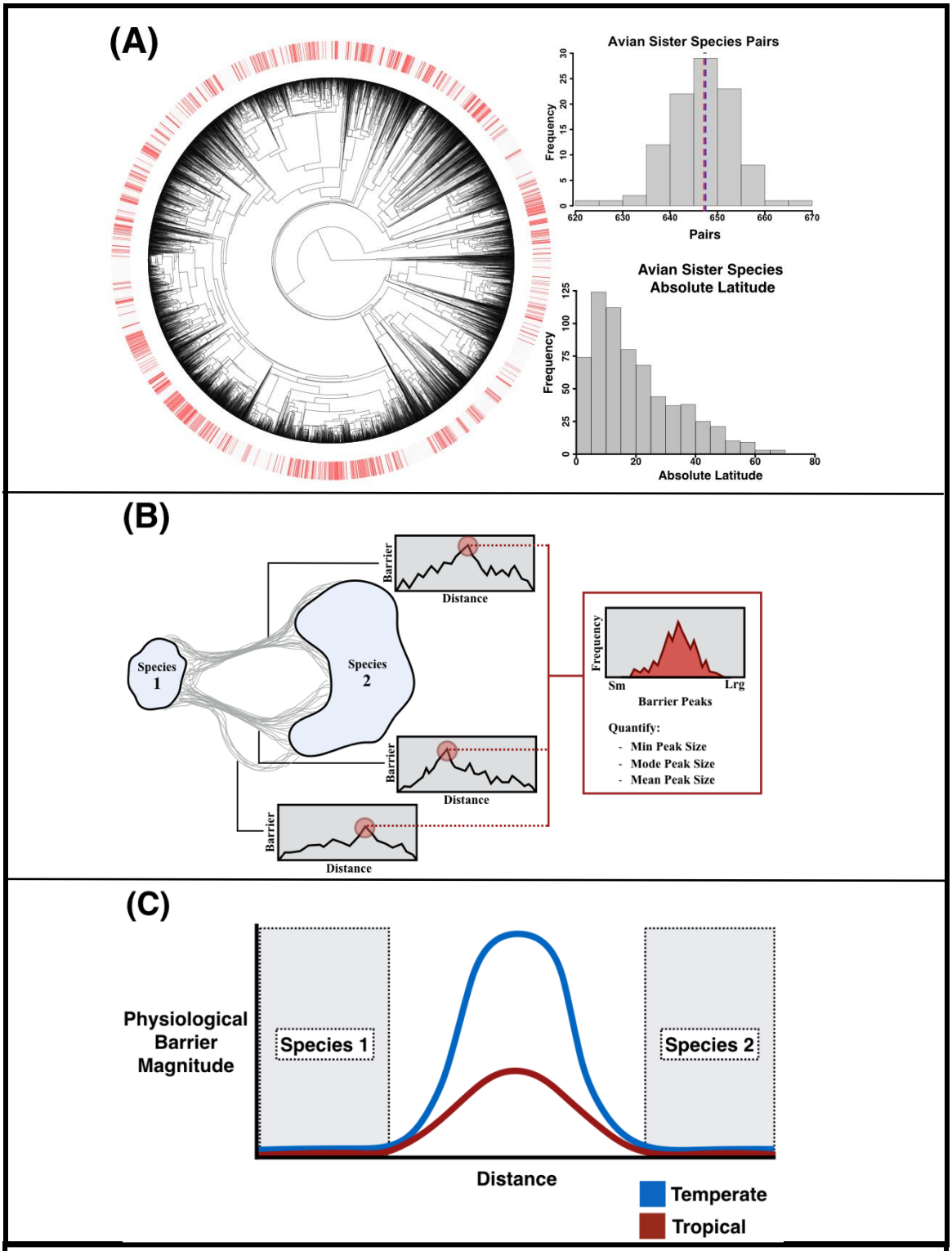


Figure 3.1. Conceptual framework for Aim 3. (A) Full avian phylogeny. Red lines mark sister species pairs. Inset: avian sister species absolute latitude, measured from midpoint between species geographic range centroids. (B) Quantification of barrier magnitude. Ranges of species 1 and species 2 depicted as irregular polygons, lines connecting polygons are paths of least resistance between all border cells of sister species ranges. Border cells with paths that cross same species range are not included. Grey barrier by distance plots depict physiological barrier magnitude profile of individual paths. Frequency by barrier peak plot depicts distribution of maximum barrier size from each path profile. Physiological barrier magnitude will be quantified from this frequency plot. (C) Physiological barrier magnitude prediction. Smaller barriers can drive speciation in the tropics; therefore, physiological barriers that separate the geographical ranges of tropical sister species will be smaller than those that separate temperate sister species pairs. Figure produced in R (R Core Team, 2017) using package 'diversitree' (FitzJohn, 2012) and 'ape' (Pardis, 2004).

Methods.

Statistical Analysis. I will analyze my hypotheses using spatial auto-regressive models at the minimum spatial resolution available for the species geographic range and environmental datasets. The response variable in all models will be thermal barrier magnitude for H1 and species richness for H2. I will run single and multiple predictor models, and I will use principle component analyses if multiple predictor variables prove to be colinear. I will rerun all models with bioregion as a random effect. I will use an additional set of models to evaluate Janzen's assumptions at the bioregion level. The response variable in all models will be mean thermal barrier magnitude (or another measure that captures among region variation in thermal barrier magnitude) for H1 and regional species richness for H2. I will run single and multiple predictor models using region values, and I will use principle component analyses if multiple predictor variables prove to be colinear. I will test H1 and H2 for all species combined and for each taxonomic group (birds, mammals, amphibians) individually. To assess the effects of environmental shielding, I will run analyses for all species combined, using only species that avoid environmental extremes (e.g., through seasonal migration or hibernation), and using only species that experience the full range of temperature seasonality. For richness analyses, I will define species membership in a bioregion in three ways: all taxa (species have membership in any bioregion where they occur), only residents (species have membership in only the bioregion where the majority of their range occurs), and only endemics (only include species with ranges that fall completely within a single bioregion) (Jetz & Fine, 2012). I will repeat all physiological barrier and richness analyses using additional axes of environmental variation (e.g., precipitation and productivity) to better understand the generality of Janzen's physiological barrier hypothesis when applied to other environmental gradients.

Environmental datasets.. I will obtain environmental data in the form of rasters from the WorldClim Version2 database (http://worldclim.org/version2; Fick & Hijmans, 2017; Hijmans et al., 2006). environmental data in this data set originate from individual weather stations globally, and are subsequently spatially interpolated to fill low coverage areas between weather stations. At a minimum, I will obtain average monthly temperature (minimum temperature, maximum temperature, and average temperature) and precipitation (monthly precipitation) data for 1970-2000, from which it is possible to calculate 19 bioclimatic variables that capture different aspects of temperature and precipitation variability, as well as measures that combine aspects of both temperature and precipitation (e.g., mean temperature of wettest quarter). datasets are also available for solar radiation, wind speed, and water vapor pressure; I may choose to include these variables in my analyses in addition to the standard temperature and precipitation values if appropriate.

Geographical datasets. I will consider only terrestrial locations in my analyses, excluding isolated islands and island chains as well as all oceanic and other aquatic locations. I will obtain elevation data in the form of 33 tiled raster files from the GTOPO 30 data set (https://lta.cr.usgs.gov/GTOPO30). This data set is a global digital elevation model (DEM) with 30 arc second (~1km) resolution that spans the full extent of latitude and longitude globally. Additionally, when necessary, I will identify mountains following the Global Mountain Biodiversity Assessment (GMBA) definition, and I will use mountain/ mountain range polygons delineated in Körner et al. (2017).

Bioregions. I will obtain bioregion data, in the form of shape files, from Olson et al. (2001), using the most recently updated, August 2012, version of the publication (https://www.worldwildlife.org/publications/terrestrial-ecoregions-of-the-world). This data set identifies 825 terrestrial ecoregions, classified into 14 different biomes (tropical and subtropical moist broadleaf forests, tropical and subtropical dry broadleaf forests, tropical and subtropical coniferous forests, temperate broadleaf and mixed forests, temperate coniferous forests, boreal forests/taiga, tropical and subtropical grasslands savannas and shrublands, temperate grasslands savannas and shrublands, flooded grasslands and savannas, montane grasslands and shrublands, tundra, Mediterranean forests woodlands and scrub, desserts and xeric shrublands, and mangroves) and 8 biogeographic realms (Nearctic, Palearctic, Neotropic, Afrotropic, Indo-Malay, Australasia, Antarctic, Oceania). There are many ways to combine the 825 terrestrial ecoregions into smaller, more inclusive, groups. In my initial analyses, I will adopt the framework used by Jetz & Fine (2012) to reduce ecoregions to a smaller set of bioregions that represent main subdivisions of the world, taking into account vegetation type (tundra, desert, grassland, boreal forest, temperate forest, tropical moist/wet forest, tropical dry forest/savanna, and Mediterranean forest/shrublands) and major land mass (nearctic, palearctic, neotropic, australasia, Indo-Malay, and afrotropic). I will, however, include the montane regions omitted by Jetz & Fine because these regions are particularly relevant to the physiological barrier hypothesis. These 32+ geographically and climatically distinct bioregions delimit largely independent 'evolutionary arenas' characterized by in situ speciation and extinction. Additionally, species tend to occur in just one bioregion and all bioregions are the scale at which allopatric speciation in vertebrates is typically proposed to occur (Jetz & Fine, 2012). Furthermore, the use of large,

independent bioregions with distinct biotas reduces geographically non-random patterns of pseudoreplication and autocorrelation, and solves problems associated with the overrepresentation of large-range species that can complicate analysis at smaller spatial grains (Jetz & Fine, 2012; Belmaker & Jetz, 2015). Organization of ecoregions into the 32+ bioregions described in Jetz & Fine, (2012) may not be the most appropriate bioregion delineation scheme for my analyses. I will therefore explore additional options for combining the 825 terrestrial ecoregions into meaningful higher-order groupings and I will consider other region delineation schemes (e.g., biomes defined by temperature and precipitation predictability; Jiang et al. 2017).

Phylogenies. I will obtain and use the most up to date species-level supertree phylogenies for mammals, birds, and amphibians. I will obtain the mammalian phylogeny from Kuhn et al. (2011); this updated tree builds on the trees created by Bininda-Emonds et al. (2008) and Fritz et al. (2009). I will obtain the avian phylogenetic tree from Jetz et al. (2012). The avian tree is particularly high-quality; it includes a very high proportion of extant avian species and includes 1000 well supported tree iterations that provide a means to account for phylogenetic uncertainty. Finally, I will obtain the amphibian phylogeny from Pyron & Wiens (2011). Before conducting any downstream analyses, I will prune the phylogenetic trees to only include the species for which geographic range maps are available. This step may reduce the number of tips on the amphibian phylogenetic tree below 25% of all extant amphibian species (full tree only has 40% of extant species before pruning). If pruning does reduce the sample below 25% of extant amphibian species I will need to find an additional source for amphibian phylogenetic information or I will need to restrict my analyses to mammalian and avian taxa.

Species geographic ranges. I will define mammalian, avian, and amphibian species geographic ranges (distributions) using IUCN range maps, rather than point localities. This is a standard definition of species geographic ranges in large-scale macroecological studies; while point localities and range maps each have their own strengths and weaknesses, range maps are generally thought to be more appropriate in studies similar to my own. IUCN range maps are in the form of spatially explicit polygons. If the data are readily available, I will use alternative range maps for birds, compiled in Belmaker & Jetz (2011), and updated in Jetz et al. (2012).

Species environmental regimes. I will generate data matrices representing the environmental regimes of all species by overlaying environmental rasters with species geographic range polygons and extracting the environment values for each grid cell in each species range (Bonetti & Wiens, 2014). Each species environmental regime will be represented by several data matrices; I will use these matrices to describe species environmental niches. I will check species environmental regime matrices for anomalous values and clean the data appropriately before proceeding.

Environmental calculations. I will estimate environmental variables for all grid cells, ecoregions, and bioregions from bioclimatic data. I will calculate seasonality, annual/mean, and annual range for temperature, precipitation, and productivity. I will calculate annual diurnal range for temperature only. I will calculate these variables for individual grid cells at the minimum spatial resolution of the environmental data. And, I will calculate mean or median values across all grid cells in ecoregions and bioregions to produce single mean/ median values for each region. The notation '*(cs)*' indicates a calculation that captures some aspect of environmental seasonality.

Temperature:

Mean temperature (BIO1): Mean of mean monthly temp.
(cs) Temperature seasonality (BIO4): Monthly variation in mean temp. (Grünig et al., 2017)
(cs) Annual temperature range: Mean temp. warmest month – Mean temp. coldest month
(cs) Annual diurnal temperature range (BIO7): Max temp. warmest month – Min temp. coldest month

Precipitation:

Annual precipitation (BIO12): Sum of monthly precip.
(cs) Precipitation seasonality (BIO15): Monthly variation in precip. (Grünig et al., 2017)
(cs) Annual precipitation range (BIO16 - BIO17): Precip. wettest quarter – Precip. driest quarter

Productivity:

Mean productivity: Mean of monthly prod.
Annual productivity: Sum of monthly prod.
(cs) Productivity seasonality: Monthly variation in prod.
(cs) Annual productivity range: Prod. most productive quarter – Prod. least productive quarter.

Species environmental niche quantification. I will describe species environmental niches from bioclimatic datasets and species geographic distributions. I will describe environmental niches for temperature, precipitation, and productivity. Because multiple aspects of environment change with elevation, I will also estimate species elevational ranges and mean elevations. In this section, maximum, minimum, and mean refer to maximum, minimum, and mean values as calculated across all grid cells in a species geographic range. I present several options for each environmental variable; the notation '*(nb)*' indicates a calculation that captures some aspect of niche breadth/ width.

Temperature:

Mean temperature: mean of annual mean temp.
(nb) Annual temperature range: Max mean temp. of warmest month – min mean temp. of coldest month
(nb) Annual diurnal temperature range: Max max temp. of warmest month – min min temp. of coldest month (Kozak & Wiens, 2007)

Precipitation:

Mean precipitation: mean of annual precip.
(nb) Annual precipitation: Max annual precip. – min annual precip. (Bonetti & Wiens, 2014)
(nb) Annual precipitation range: Max precip. of wettest quarter – min precip. of driest quarter (Bonetti & Wiens, 2014)

Productivity:

Mean productivity (1): mean of annual mean prod.
Mean productivity (2): mean of annual prod.
(nb) Annual productivity: Max annual prod. – min annual prod.
(nb) Annual productivity range: Max prod. most productive quarter – min prod. least productive quarter

Elevation:

Mean elevation: mean of elevation
(nb) Elevational range: max elev. – min elev. (Kozak & Wiens, 2007)

Identification of sister species pairs. I define sister species pairs as pairs of species that are each other's closest relatives. I will identify sister species pairs in the avian, mammalian, and amphibian phylogenies as cherry pairs, or "pairs of leaves each of which is adjacent to a common ancestor" (McKenzie & Steel, 2000) using the 'diversitree' package in R (R Core Team, 2017).

Barrier magnitude quantification. I will measure physiological barrier magnitude as the size of the environmental barrier that separates the geographic ranges of the two members of a sister species pair. I will define physiological barriers separately for temperature, precipitation, and productivity for all species. I will first connect the border grid cells of the two species ranges using the paths of least resistance (trade-offs between barrier magnitude and distance will be used to determine least resistance path) and river-flow modeling techniques. I will then build barrier profiles for each path connecting species ranges and I will extract the largest physiological barrier value along each path. These maximum barrier values will be used to build a barrier magnitude frequency profile for each species. I will consider the minimum, mean, and mode barrier magnitude from each barrier magnitude frequency profile. These final values will be used in statistical analyses whenever physiological barrier magnitude is mentioned.

$$[3.1]\ Environmental\ Difference = \sum_{i=1}^{12} |M_{Ai} - M_{Bi}|$$

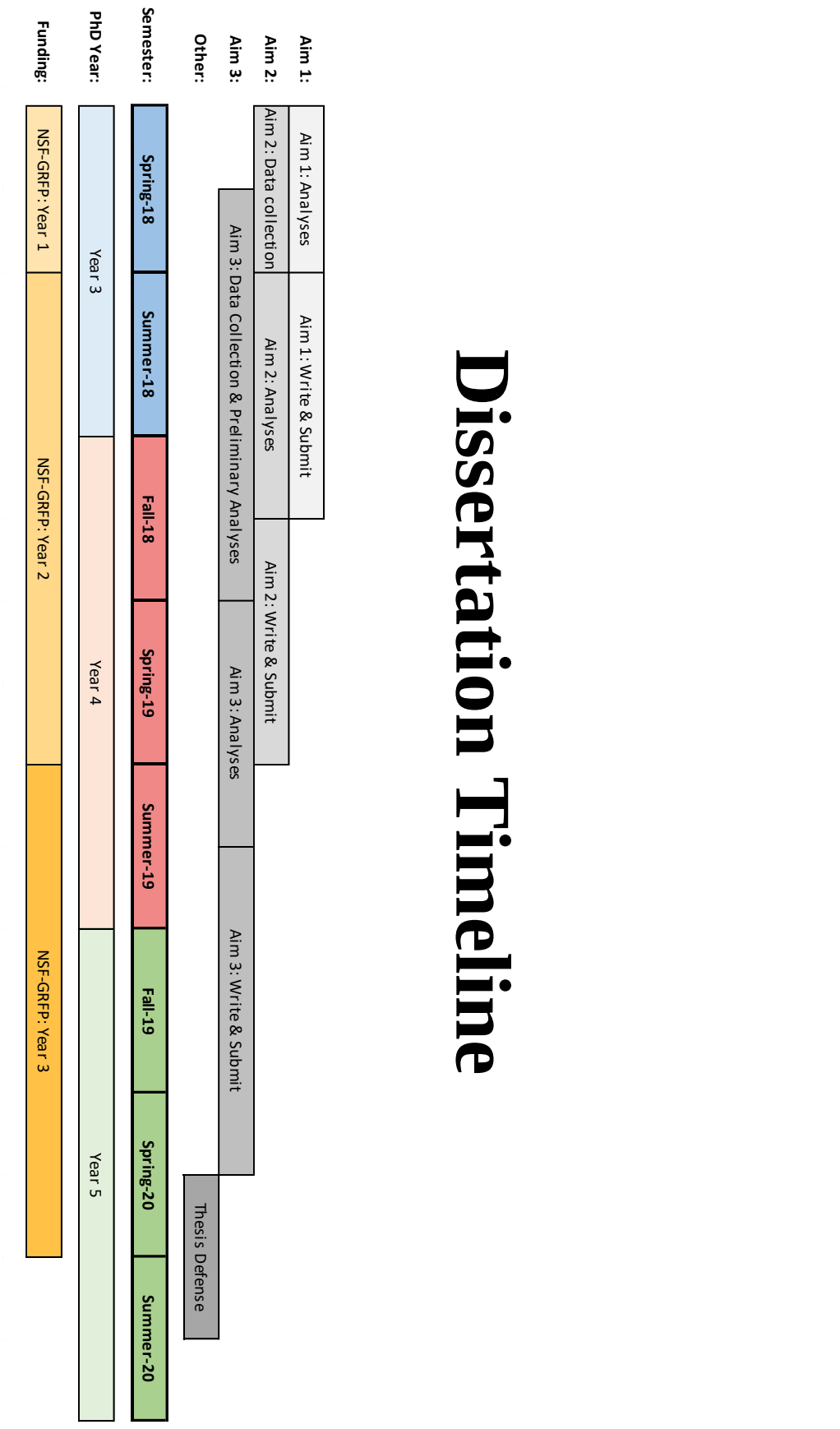
Equation 3.1 Enviornmental difference between two climate regimes. M_{Ai} and M_{Bi} are the mean environment values for regime A and B, for month *i*. environmental difference can be calculated between grid cells at the minimum resolution of the environmental data and at coarser grains (110km x 110km grid cells, ecoregions/bioregions, species climate regimes) using average values to calculate M_A and M_B.

$$[3.2a]\ Environmental\ Overlap = \sum_{i=1}^{12} \frac{o_i}{\sqrt{R_{Ai}R_{Bi}}}$$

$$[3.2b]\ Environmental\ Overlap = \sum_{i=1}^{12} 0.5\left(\frac{o_i}{R_{Ai}} + \frac{o_i}{R_{Bi}}\right)$$

Equation 3.2. environmental overlap between two environmental regimes. R_{Ai} and R_{Bi} are the environmental ranges present in regime A and B, for month *i*. o_i is the overlap of R_A and R_B (in degrees Celsius) for month *i*. For equation (3.2a) monthly overlap can have a maximum value of 1 (environmental regimes are identical) and annual overlap can have a maximum value of 12; note that complete inclusion of one regime within the other does not produce maximal overlap. There are no lower bounds on monthly or annual overlap values in version (3.2a). For equation (3.2b) Monthly overlap values range from 0 to 1 and the total annual environmental overlap value ranges from 0 to 12. environmental overlaps can be calculated between grid cells at the minimum resolution of the environmental data and at coarser grains (110km x 110km grid cells, ecoregions/bioregions, species environmental regimes) using average values to calculate R_A and R_B. Equation (3.2a) modified from Janzen (1967); equation (3.2b) first published in Kozak & Wiens (2007).

Quantify Species richness. I define species richness as the number of different species represented in a 110km x 110km grid cell or bioregion. This measure of diversity does not consider the abundance or relative abundance of species. I will calculate Bioregion species richness in three ways: total richness, count of all species that occur in a bioregion; resident richness, count of all species that occur primarily in the focal bioregion; and endemic richness, count of all species that occur only in the focal bioregion. I will only consider total richness at the 110km x 110km scale, because the other measures have little biological meaning when the areas of comparison do not represent separate evolutionary arenas.


Dissertation Timeline
Aim 1:
Aim 2:
Aim 3:
Other:
Semester:
PhD Year:
Funding:
Aim 1: Analyses
Aim 1: Write & Submit
Aim 2: Data collection
Aim 2: Analyses
Aim 2: Write & Submit
Aim 3: Data Collection & Preliminary Analyses
Aim 3: Analyses
Aim 3: Write & Submit
Thesis Defense
Spring-18
Summer-18
Fall-18
Spring-19
Summer-19
Fall-19
Spring-20
Summer-20
Year 3
Year 4
Year 5
NSF-GRFP: Year 1
NSF-GRFP: Year 2
NSF-GRFP: Year 3

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