Diverse genotypes of the amphibian killing fungus produce distinct phenotypes through plastic responses to temperature

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DATA ACCESSIBILITY

Raw data and R code is available from figshare: https://doi.org/10.6084/m9.figshare.7371191.

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ABSTRACT

Phenotypes are the target of selection and affect the ability of organisms to persist in variable environments. Phenotypes can be influenced directly by genes and/or by phenotypic plasticity. The amphibian-killing fungus *Batrachochytrium dendrobatidis* (Bd) has a global distribution, unusually broad host range, and high genetic diversity. Phenotypic plasticity may be an important process that allows this pathogen to infect hundreds of species in diverse environments. We quantified phenotypic variation of nine Bd genotypes from two Bd lineages (Global Pandemic Lineage [GPL] and Brazil) and a hybrid (GPL-Brazil) grown at three temperatures (12, 18, and 24 °C). We measured five functional traits including two morphological traits (zoospore and zoosporangium sizes) and three life history traits (carrying capacity, time to fastest growth, and exponential growth rate) in a phylogenetic framework. Temperature caused highly plastic responses within each genotype, with all Bd genotypes showing phenotypic plasticity in at least three traits. Among genotypes, Bd generally showed the same direction of plastic response to temperature: larger zoosporangia, higher carrying capacity, longer time to fastest growth and slower exponential growth at lower temperatures. The exception was zoospore size, which was highly variable. Our findings indicate that Bd genotypes have evolved novel phenotypes through plastic responses to temperature over very short time scales. High phenotypic variability likely extends to other traits and may facilitate the large host range and rapid spread of Bd.

Keywords: pathogen, disease ecology, climate, amphibians, phylogenetic conservatism, phenotypic plasticity, chytrid, *Batrachochytrium dendrobatidis*

INTRODUCTION

Organisms cope with changing environments through phenotypic plasticity, allowing species to move into new habitats and persist in current habitats through changing seasons and climates (Desprez-Loustau et al., 2007, Matesanz et al., 2010, Nicotra et al., 2010). Phenotypic plasticity is the capacity of a genotype to express different phenotypes in response to different environmental conditions and is subject to evolution by natural selection and other evolutionary mechanisms (Bradshaw, 1965, West-Eberhard, 1989, West-Eberhard, 2005, Ghalambor et al., 2007). Phenotypic plasticity can be distinguished from local adaptation (e.g., genetic differentiation) by housing individuals with the same genotype under different environmental conditions in common garden experiments (Dorman et al., 2009, Pelini et al., 2012). Over the last several decades, phenotypic plasticity has received considerable attention from ecologists and evolutionary biologists (Bradshaw, 1965, Foster, 1979, West-Eberhard, 1989, Pigliucci et al., 2006, Zamudio et al., 2016) as it is one mechanism that may facilitate or accelerate the process of adaptive evolution through genetic accommodation (Pigliucci et al., 2006, West-Eberhard, 2005, Gomez-Mestre & Buchholz, 2006, Ghalambor et al., 2007). Even plasticity that is not currently adaptive can provide sources of novel phenotypes important in trait evolution (Lande, 2009, Nicotra et al., 2010) demonstrating the value of phenotypic plasticity independent of adaptive potential.

Given that it is not feasible to assess plastic responses for all phenotypic traits, it is important to identify functional traits to target (Nicotra et al., 2010). Functional traits are traits that impact organism performance or fitness, and can be morphological, physiological or behavioral characteristics such as: organism height or size, salt tolerance and maximum growth rate (McGill et al., 2006, Green et al., 2008, Gravel et al., 2016). Plasticity in functional traits is visualized through reaction norms, which characterize how genotype, environment, and genotype by environment interactions yield specific phenotypes. Reaction norms allow us to predict how shared ancestry and

environment influence an organism's response to changing environments, whether temporally or spatially (Scheiner, 1993, Ghalambor et al., 2007). Predicting organismal responses is important because the ability of many species to cope with global change or invade new habitats is related to the current amount of plasticity in their functional traits (Desprez-Loustau et al., 2007, Matesanz et al., 2010, Nicotra et al., 2010).

The integration of phenotypic plasticity, phylogenetic relationships, and functional traits can reveal the relative importance of shared ancestry and environmental conditions on trait evolution (Figure 1, (Pigliucci et al., 1999, Pollard et al., 2001, Burns & Strauss, 2012, Lennon et al., 2012, Relyea et al., 2018). Closely related organisms can resemble each other more closely in a functional trait than expected by chance, a signal known as phylogenetic trait conservatism, and can reflect strong stabilizing selection or a low rate of evolutionary change (Revell et al., 2008, Martiny et al., 2013, Davies et al., 2013). For instance, strong phylogenetic conservatism of phenotypic traits associated with pathogen virulence would have the practical benefit of allowing the prediction of virulence from genotype data (Fisher et al., 2009). Alternatively, a functional trait can be dissimilar among closely related organisms, known as evolutionary lability, and can reflect rapid evolutionary change via local adaptation or genetic drift (Revell et al., 2008, Zhang et al., 2017). In cases of evolutionary lability, taxa may show the same pattern of plasticity to different environments revealing a response that is predictable based on environmental conditions, but not evolutionary relationships (Figure 1, Piglucci et al., 1999).

Fungi compensate for simple structural body plans by using genetic and phenotypic variation to adapt to changing environments (Sylvia et al., 2005, Angelard et al., 2014, Muggia et al., 2014). A prime example is the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), a pathogen that has caused population declines of amphibians globally (Lips et al., 2006, Skerratt et al., 2007). Bd has a simple two-stage life cycle consisting of motile zoospores developing into encysted zoosporangia, which produce new zoospores. Bd is comprised of multiple lineages, which collectively have a worldwide distribution and high genetic diversity. Bd diversity includes genetically distinct and geographically-restricted lineages of Bd in South Africa (Bd-CAPE), Brazil and Asia (Bd-Brazil/Asia-2, and Bd-Asia1) as well as the globally distributed and hyper-

virulent global panzootic lineage (Bd-GPL) (Schloegel et al., 2012, Rosenblum et al., 2013, James et al., 2015, O'Hanlon et al., 2018). Bd-GPL is the primary lineage associated with catastrophic mass mortalities, rapid population declines, and species extinctions of amphibians globally (Farrer et al., 2011, Olson et al., 2013, James et al., 2015). Bd has an unusually broad host range, infecting hundreds of amphibian species (Olson et al., 2013). A major question is how did Bd become so widely distributed among diverse host species and environments; especially given that Bd sexual reproduction is extremely rare (Berger et al., 2005, Schloegel et al., 2012). Phenotypic plasticity may play an important role in adaptation to new environments during the spread and evolution of Bd. For instance, phenotypic traits of Bd morphology and life history have been associated with Bd virulence. Specifically, large Bd zoospore size, large zoosporangium size, slow growth rate and high carrying capacity have been correlated with higher Bd infection loads and higher amphibian mortality rates (Fisher et al., 2009, Voyles, 2011, Piovia-Scott et al., 2015, Lambertini et al., 2016, Becker et al., 2017, Voyles et al., 2017). Yet, the basis of Bd intraspecific phenotypic trait variation and the link to its worldwide distribution are still poorly understood.

We quantified functional trait plasticity of nine *Bd* genotypes at three temperatures (12, 18 and 24 ˚C) using a common garden experiment and examined the results in a phylogenetic framework. Temperature is known to affect Bd phenotypic traits *in vitro* (Piotrowski et al., 2004, Woodhams et al., 2008, Stevenson et al., 2013) and *in vivo* (Berger et al., 2004, Woodhams & Alford, 2005, Kriger et al., 2007, Longo et al., 2010, Sapsford et al., 2013). Yet, our understanding of temperature-induced phenotypic changes in functional traits across Bd genotypes is limited. Our first objective was to determine how temperature, genotype and their interaction affected the expressed phenotype for five functional traits, including two morphological (zoospore and zoosporangium size) and three life history traits (carrying capacity, time to fastest growth, and exponential growth rate). Our second objective was to quantify the role of evolutionary history in phenotypic trait responses to temperature for the five functional traits. The findings from these objectives are important for understanding seasonal disease dynamics, the spread of this pathogen into new environments and future disease dynamics.

MATERIALS AND METHODS

Experiment design

We grew isolates from nine Bd genotypes (Table 1) at three temperatures in 96-well plates using a full factorial design. We selected three temperatures (12, 18 and 24 ˚C) within the temperature range for Bd growth (Piotrowski et al., 2004, Stevenson et al., 2013, Voyles et al., 2017). We selected isolates that were genotyped in previous studies (Schloegel et al., 2012, Jenkinson et al., 2016) and represented genetic diversity within and among Bd lineages, including the Bd-Brazil lineage, a Bd-Brazil-GPL hybrid, and several Bd-GPL genotypes.

To prepare Bd genotypes for the experiment, we passaged cryopreserved isolates (Boyle et al., 2003) on 1% tryptone agar plates twice, and then grew them on 1% tryptone agar plates for six days at 18 ˚C. Prior passage history was minimal for all isolates (3-9 passages), except for GPL-JEL258 (26 passages). We harvested zoospores by flooding plates with 1% tryptone broth, letting them sit for 20 minutes, and filtering the solution through a sterile 11 µm filter to remove thalli. We counted zoospore density with a Bright-Line hemocytometer and diluted harvested zoospores to 1 x 10^6 zoospores/mL with 1% tryptone broth.

We set-up a total of eighteen 96-well plates for the experiment. We randomly assigned Bd genotypes to a column on each plate (n = 8 wells per Bd genotype per plate) with negative control wells in columns 1 and 12. To set up the 96-well plates, we added 100 µl of the designated Bd isolate to the experimental wells (approximately 1 x 10^5 zoospores) and 100 µl of 1% tryptone broth to the negative control wells. We sealed the plates with parafilm and placed three plates into one of six environmental chambers (Percival model DR-36VL) set to 12, 18 or 24 ˚C (two chambers per temperature). Each environmental chamber housed three 96-well plates. We destructively sampled individual wells from one plate to measure two morphological traits (zoospore and zoosporangium size) over time. We used the two other plates to repeatedly measure cell density over time to estimate three life history traits (carrying capacity, time to fastest growth, exponential growth rate) during the 16-day experiment.

Zoospore and zoosporangium size measurement

We quantified zoosporangium and zoospore size (n = 18 per life stage), defined as the area of the cell's cross-section. Prior to the experiment, we conducted a pilot study to determine the maximum number of Bd isolates that we could measure in a single day and the optimal day (e.g., the day when zoospores were being released) to measure size at each temperature (see Supplementary Information). We determined that approximately nine was the maximum number of genotypes that we could prepare and measure in an 8-hour time period. We sampled plates for two consecutive days, with the first day of sampling dependent on the temperature. The first day was selected for zoosporangium size measurements and based on our pilot study observations represented the period of maximum zoosporangia maturity. The following day we measured zoospore size and based on our pilot study observations represented the maximum period of zoospore release. In the experiment, we measured zoosporangium size on Day 2 for 24 ˚C, Day 3 for 18 ˚C, and Day 6 for 12 ˚C. Zoospores were measured on Day 3 for 24 ˚C, Day 4 for 18 ˚C, and Day 7 for 12 ˚C. For each sampling day, we randomly chose a row per plate on each of six plates to measure each genotype. We scraped the bottoms and sides of the wells with a micropipette tip to dislodge cells and transferred all 100 µl of Bd solution to separate micro-centrifuge tubes. We kept tubes on ice until imaging. We imaged cells using DIC microscopy with a Zeiss AxioPhot light microscope capturing images using a CoolSNAP EZ CCD camera. We imaged wet-mounts of zoosporangia at 400x total magnification and zoospores with oil immersion at 1000x total magnification. We identified zoospores and zoosporangia along a Z-shaped transect starting in the upper left corner of the slide and ending in the lower right corner. We photographed the first nine fields of view per slide that contained at least one zoosporangium or zoospore matching our criteria (Figure S1). Our criteria for mature zoosporangia were visible rhizoids, no flagella, no single internal vesicle, and no release of internal zoospores. Our criteria for zoospores were presence of flagella, absence of any rhizoids or large internal compartments, and free from parent zoosporangia. We traced the border of the largest zoosporangium or zoospore in the field of view using ImageJ64 version 1.47 (Rasband, 2014) and

calculated the area of the cell cross-section. Each Bd-temperature combination had a total of 18 measurements per life stage.

Growth curve measurements & life history traits

We measured Bd cell optical density (OD) at 492 nm wavelength using a Biotek spectrophotometer (Model ELx800). Optical density is used as a measure of the concentration of a microorganism in suspension. For each well in the twelve 96-well plates (four plates per temperature), we measured OD every other day starting on inoculating day (Day 0) and ending on Day 16. Each genotype-temperature combination had a total of 32 well readings per day.

We quantified three Bd life history traits by fitting logistic growth models to OD measurements (Piovia-Scott et al., 2015). We fit a logistic model to replicate wells of each genotype on each plate using function *nls* in the package 'stats' giving a total of 108 separate equations (9 genotypes x 3 temperatures x 4 plates/temperature).

$$y = \frac{asym}{1 + e^{\frac{(xmid - t)}{scal}}}$$

Here, *y* is Bd cell OD, *t* is time in days, *asym* is the top horizontal asymptote and represents the carrying capacity, *xmid* is the time point where population density is half of *asym*, and represents the time to fastest growth, and *scal* is the inverse of the slope of growth at xmid and 1/scal represents the exponential growth rate (Caroli et al., 2010).

Statistical analysis

We used R version 3.4.1 (R-Core-Team, 2017) for all statistical analyses, and used the package 'ggplot2' for generating figures (Wickham, 2009). All R code and raw data files are accessible at figshare, *and will be uploaded following acceptance of the manuscript*.

We determined the effects of temperature, genotype and their interaction on zoospore and on zoosporangium sizes using a separate linear mixed-effects model for each morphological trait (response variable). To achieve a normal distribution, we used a square-root transformation of zoospore and zoosporangium sizes. We used the *lmer* function in the package 'lme4' (Bates et al., 2015) to run the models, and included

incubator as a random effect in each model. We used a likelihood ratio test to determine the significance of variables using the *anova* function in the package 'stats' (R-Core-Team, 2017). We performed *post hoc* analyses using the *lsmeans* function in the package 'lsmeans' (Lenth, 2016) and identified size groups from pairwise comparisons using the *cld* function in the package 'lme4'.

We determined the effect of temperature, genotype and their interaction on three life history traits using a separate linear mixed-effects model for each parameter estimate from the logistic models (asym, xmid, and 1/scal, n = 108 estimates per trait; response variable). We used the *lmer* function to run the models, the *anova* function to determine significance and the *lsmeans* function for *post hoc* analyses as above, and included plate nested within incubator as random effects in each model. From *post hoc* analyses, we identified temperatures that had high variability among Bd genotypes by examining the number of significant pairwise comparisons between temperatures.

We tested for a phylogenetic signal in phenotype and phenotypic plasticity among Bd genotypes using Blomberg's *K* statistic (Blomberg et al., 2003). Testing phylogenetic signal with nine taxa increases Type II error rate compared to larger trees (Freckleton et al., 2002, Blomberg et al., 2003). In our analyses, detecting phylogenetic dependence of traits would indicate phylogenetic signal, but not detecting phylogenetic dependence could relate to a lack of power. To construct the phylogenetic tree of the Bd lineages, we used the neighbor-joining algorithm in PAUP*4.0 based on concatenated multilocus genotypes for 36 loci from multiple chromosomal regions (Schloegel et al., 2012, Jenkinson et al., 2016), midpoint rooted the tree using the function *midpoint.root* in the package 'phytools' (Revell, 2012), and transformed the topology to an ultrametric tree using the function *chronos* in the package 'ape' (Paradis et al., 2004). For phenotypic trait values, we used the mean parameter estimate from the morphological size and growth models (Table S1). We tested for an association between traits and phylogeny with each mean trait value (e.g. zoospore size at 12℃) using the function *multiPhylosignal* in the package 'picante' (Kembel et al., 2010). Traits with p-values for phylogenetically independent contrast variance (PIC.variance.p) < 0.05 indicate that traits have a conserved phylogenetic signal indicating phylogenetic trait conservatism. For phenotypic plasticity, we calculated the relative distance plasticity index (RDPI;

(Valladares et al., 2006) for each trait using the function *rdpi* in the package 'Plasticity' (Ameztegui, 2017). We then tested for an association of phylogeny with each mean RDPI value using the function *multiPhylosignal* in the package 'picante' (Kembel et al., 2010).

RESULTS

Plastic, but predictable responses in functional traits

We found that all functional traits showed statistically significant genotype by temperature interactions (Table 2). Likewise, phenotypic plasticity (temperature effect) was statistically significant for all traits. The details of the reaction norms differed dramatically from trait to trait and from genotype to genotype (Figures 2 and 3). Yet, all traits except for zoospore size, showed a generally consistent pattern of phenotypic plasticity among genotypes (Figures 2 and 3). When genotypes showed phenotypic plasticity they generally maintained the same direction of the plastic response for that trait. For instance, Bd genotypes generally produced larger zoosporangia at lower temperatures (Figure 2b) and grew slower at lower temperatures (Figure 3b).

The early developmental trait, zoospore size, showed highly variable plasticity and a strong genotype by temperature interaction (LMM: $X^2_{(16,\ n = 486)} = 41.1$, $p < 0.001$, Figure 2a, Table 2). Five of the nine genotypes displayed phenotypic plasticity (Figure 2a, Table S2, LMM temperature effect: $X^2_{(2,\ n = 486)} = 7.3$, $p = 0.03$, pairwise $p < 0.05$), with three genotypes producing larger zoospores at 12 ˚C than at 18 ˚C (GPL-JEL647, GPL-CLFT023, and GPL-SRS810), and two genotypes producing larger zoospores at 24 ˚C compared to 12 ˚C (GPL-JEL258 and GPL-PAB001). Some genotypes were consistently larger in zoospore size (GPL-CLFT026 and GPL-JEL258) compared to other genotypes, while others were consistently smaller (Brazil-JEL649 and GPL-SRS810) across temperatures (Figure 2a, LMM genotype effect: $X^2_{(8,\ n = 486)} = 119.6$, $p < 0.001$, pairwise $p < 0.05$).).

Mature zoosporangium size, marking the switch to reproductive phase, was also variable, but to a lesser extent than zoospore size (Figure 2). Unlike zoospore size, the genotypes showing zoosporangium size plasticity displayed a conserved response to

temperature, except for GPL-CLFT023. Specifically, we found that five genotypes showed phenotypic plasticity (GPL-JEL258, GPL-JEL647, GPL-JEL423, GPL-SRS810, Hybrid-CLFT024.2) with those genotypes consistently producing the largest zoosporangia at 12˚C (Figure 2b, Table S2, LMM temperature effect: $X^2_{(2, n = 486)} = 8.6$, $p = 0.01$, pairwise $p < 0.05$). For instance, GPL-JEL258 showed the largest change in zoosporangium size between temperatures, increasing in area by 191.2 μm^2 from 24 ˚C to 12 ˚C. GPL-PAB001 was consistently larger in zoosporangium size than other genotypes, while Brazil-JEL649 and GPL-CLFT023 were consistently smaller across temperatures (Figure 2b, LMM genotype effect: $X^2_{(8, n = 486)} = 120.8$, $p < 0.001$, pairwise $p < 0.05$).

Bd genotypes generally grew more rapidly with increasing temperature, but they reached a lower carrying capacity at higher temperatures (Figure 3). All nine Bd genotypes showed phenotypic plasticity in carrying capacity (Figure 3a, Table S2, LMM temperature effect: $X^2_{(2, n = 861)} = 26.8$, $p < 0.001$, pairwise $p < 0.05$), with an increased carrying capacity at lower temperatures except Brazil-JEL649 (highest carrying capacity at 18 ˚C). All nine Bd genotypes showed phenotypic plasticity in days to fastest growth (Figure 3b, Table S2, LMM temperature effect: $X^2_{(2, n = 861)} = 37.9$, $p < 0.001$, pairwise $p < 0.05$), with a decrease in days to exponential phase as temperature increased. Eight Bd genotypes showed phenotypic plasticity in exponential growth rate (Figure 3c, Table S2, LMM temperature effect: $X^2_{(2, n = 861)} = 29.8$, $p < 0.001$, pairwise $p < 0.05$), with the slowest growth rate at 12 ˚C for six genotypes, and at both 12 and 24 ˚C for the other two genotypes (GPL-JEL647 and Hybrid-GLFT024.2). GPL-JEL423 grew at a consistent rate across temperatures (i.e., no phenotypic plasticity). Unlike zoospore and zoosporangium size, we found no genotypes that consistently produced larger or small growth trait values across temperatures. Instead, we found that Bd growth patterns were most dissimilar among genotypes at lower temperatures and converged as temperature increased (Figure 4).

Evolutionary lability in functional traits and plasticity

We found no evidence for phylogenetic trait conservatism among genotypes in phenotypic trait values or in phenotypic plasticity indexes (PIC.variance.p > 0.05; Figures 5 and 6).

DISCUSSION

Our study offers new insight into the drivers of phenotypic variation and plasticity across multiple genotypes of the amphibian chytrid pathogen Bd. Organisms that exhibit phenotypic plasticity can rapidly change their morphology, physiological state, and other aspects of their ecology in response to environmental stimuli (Pigliucci et al., 2006, West-Eberhard, 2005). Theory predicts that phenotypically variable populations are associated with decreased vulnerability to environmental changes, increased invasive capacity, larger distribution ranges and lower risk of extinctions when compared to less phenotypically variable populations (Desprez-Loustau et al., 2007, Forsman et al., 2008, Wennersten & Forsman, 2012). For instance, four fungal plant pathogens invaded semi-arid areas, where they were not expected to survive, because of high phenotypic plasticity in penetration rate and spore survival (Bashi & Rotem, 1974). We found that Bd can evolve novel phenotypes through plastic responses to temperature over very short time scales. This high variability in temperature responses may have facilitated the enormous host range (Olson et al., 2013) and rapid global spread of Bd in the last decades (Lips et al., 2006, Skerratt et al., 2007, O'Hanlon et al., 2018).

Temperature influences Bd growth, survival and virulence. Bd generally grows slower at lower temperatures *in vitro* (Piotrowski et al., 2004, Woodhams et al., 2008, Stevenson et al., 2013), but cooler temperatures are often associated with severe chytridiomycosis outbreaks *in vivo* (Berger et al., 2004, Woodhams & Alford, 2005, Kriger et al., 2007, Longo et al., 2010, Sapsford et al., 2013). Bd may counter slow growth rate at lower temperatures with increased virulence. Virulence is the reduction in host fitness due to infection (Read, 1994). Increased virulence (e.g., higher host mortality) is associated with higher Bd infection loads (Briggs et al., 2010). Possible life history strategies that may increase virulence at lower temperatures include larger zoosporangium size and maintenance of long-term growth producing more zoospores

over longer time periods (Woodhams et al., 2008). We found that most Bd genotypes produced larger zoosporangia at lower temperatures. Larger zoosporangium size is linked to higher Bd virulence (Fisher et al., 2009, Lambertini et al., 2016), likely because larger zoosporangia are more disruptive to amphibian skin function (Greenspan et al., 2012) and produce more infectious zoospores (Stevenson et al., 2013). Second, we found a strongly conserved response of slower growth and higher carrying capacity (production of more zoospores and zoosporangia) for all Bd genotypes at lower temperatures. Producing more infectious zoospores over a longer time period is related to higher mortality in amphibians (Piovia-Scott et al., 2015, Lambertini et al., 2016, Maguire et al., 2016). Thus, Bd genotypes showed specific traits (i.e., larger zoosporangia, slower growth and higher carrying capacity) that likely make them more infectious at lower temperatures, when amphibians' immune system response is reduced (Raffel et al., 2006, Ribas et al., 2009, Longo & Zamudio, 2017).

We found that Bd genotypes displayed similar growth patterns at high temperature, but had highly variable responses at lower temperatures, likely reflecting developmental or genetic constraints and selection. All lineages (GPL, Brazil, Hybrid) showed similar growth patterns at 24 ˚C suggesting that conserved, ancestral developmental/genetic factors constrain growth responses at higher temperatures. The increased variability among genotypes at 12 ˚C could be interpreted as a consequence of the adaptive importance of temperature and suggests that Bd genotypes vary in their adaptation to cooler temperatures. Bd completes its life cycle within keratinized tissue, initially invading as zoospores a few layers deep, and then maturing into zoosporangia as the epidermal cells moves outwards and keratinize (Berger et al., 2005). During this time Bd is interacting with host skin tissue, host immune molecules, and host skin microbiomes, all of which can vary in composition among host species and environments (Ellison et al., 2014, Muletz Wolz et al., 2017, Ohmer et al., 2017). The more variable growth patterns we observed at lower temperatures may result from variation in these host and environmental pressures on Bd genotypes.

We found preliminary evidence that functional traits and their plasticity evolved independently of Bd phylogenetic relationships. Tests of phylogenetic signal are not without shortcomings and the short evolutionary divergence time of Bd-GPL and our

limited sample size for taxa may have influenced the chances of detecting a phylogenetic signal (Freckleton et al., 2002, Blomberg et al., 2003, Revell et al., 2008). We encourage future studies to focus on traits that are easy to measure within one study design and increase sample size of Bd genotypes to > 20 genotypes (Blomberg et al., 2003) from multiple Bd lineages to increase the power to test phylogenetic signal. Nonetheless, other studies have similarly found a lack of phylogenetic signal in morphological and life history trait values for Bd genotypes from single lineages (Piovia-Scott et al., 2015, Lambertini et al., 2016) and from different lineages (Fisher et al., 2009, Becker et al., 2017), supporting a general trend across multiple divergence times. One exception was found by Fisher et al., (2009), who reported that genetic distance predicted average zoosporangium size for 11 globally-distributed Bd isolates. Their study was the only one to include Bd isolates from the same genotype (5 isolates), which likely increased the probability of observing similar sizes among closely related isolates. Epigenetic changes, microevolution, and chromosomal copy number changes can occur rapidly (Reed et al., 2010, Farrer et al., 2013, Refsnider et al., 2015), and are potential mechanisms that can explain how Bd genotypes evolve phenotypic traits independent of shared ancestry. Further analyses of Bd phenotype and virulence correlates should take into account that these factors may not be explained by genotypic differences in Bd.

Pathogenic fungi rely on phenotypic and genetic variability to disperse, survive and reproduce, which impact their virulence (Garbelotto et al., 2015, Greenspan et al., 2018). Four of the five traits we measured have been linked to virulence *in vivo*. Larger Bd zoospore size, larger zoosporangium size, slower growth rate and higher carrying capacity are correlated with higher Bd infection loads and higher amphibian mortality (Fisher et al., 2009, Voyles, 2011, Piovia-Scott et al., 2015, Lambertini et al., 2016, Becker et al., 2017, Voyles et al., 2017). Bd-Brazil produced the smallest zoospores and zoosporangia across temperatures, which may explain the lower virulence and infectiveness of Bd-Brazil compared to Bd-GPL and the GPL-Brazil hybrid (Rodriguez et al., 2014, Jenkinson et al., 2016, Greenspan et al., 2018). We quantified these phenotypic traits *in vitro* as the same stages of the life cycle occur within epidermal cells of amphibian skin as in culture (Berger et al., 2005). We encourage future studies to

examine these relationships in live animal hosts. For example, we found that GPL-JEL258 produced similar size zoosporangia across temperatures and a closely related genotype GPL-CLFT026 decreased in size with temperature; the Brazilian genotype Brazil-JEL649 produced similar size zoosporangia across temperatures, but they were significantly smaller than all other genotypes. Exposing the same amphibian species to these three genotypes at a range of temperatures would allow for a test of the causal linkages among temperature, zoosporangium size plasticity, lineage and virulence. Assessing the magnitude of phenotypic plasticity *in vivo* will be essential to fully understanding complex environment-pathogen dynamics.

CONCLUSION

The number of diseases caused by pathogenic fungi and their frequency of outbreaks have both increased in the last few decades (Fisher et al., 2012). To predict and address threats from pathogens, we need to know the conditions that allow these pathogens to thrive and be able to predict how rapidly changing environments will impact pathogen dynamics. We found evidence that Bd can rapidly evolve novel phenotypes through phenotypic plasticity in response to temperature, independent of shared ancestry, but may be genetically constrained to adapt to high temperatures. It has been predicted that with climate change, the geographic range of Bd and its influence on amphibian biodiversity could be reduced (Rodder et al., 2010) and our results suggest a limit on the evolution of growth at higher temperatures. Understanding the history and plasticity of functional traits is essential for predicting how organismal ecology and evolution shape pathogen traits and their associated virulence. We found that Bd generally showed the same pattern of plasticity to temperature revealing trait responses that are predictable based on environmental conditions (but not evolutionary relationships). Future studies are warranted to relate temperature-induced phenotypic plasticity to virulence *in vivo*.

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TABLES:

Table 1. Nine *Bd* isolates used in this study. Genotype names are the composite of Lineage and Isolate ID.

Lineage	Isolate ID	Isolation Locality	Amphibian Host
Brazil	JEL649	Jundiaí, São Paulo, Brazil	*Hylodes japi*
GPL	CLFT026	Iporanga, São Paulo, Brazil	*Boana faber*
GPL	JEL258	Orono, Maine, USA	*Lithobates sylvaticus*
GPL	JEL647	Point Reyes, California, USA	*Hyliola regilla*
GPL	CLFT023	Camanducaia, Minas Gerais, Brazil	*Boana sp.*
GPL	JEL423	Guabal, Panama	*Agalychnis lemur*
GPL	PAB001	Maricao, Puerto Rico	*Eleutherodactylus coqui*
GPL	SRS810	Savannah River, South Carolina, USA	*Lithobates catesbeianus*
Hybrid	CLFT024.2	Morretes, Parana, Brazil	*Hylodes cardosoi*

Table 2. Significance of factors and interaction terms for each trait. Chi-square values are reported from the likelihood ratio tests comparing linear mixed-effect models. Significance levels: * = 0.03, ** = 0.01, *** < 0.001

	Genotype (df = 8)	Temperature (df = 2)	Genotype x Temperature (df = 16)
Zoospore size	X^2 = 119.6***	X^2 = 7.3*	X^2 = 41.1***
Zoosporangium size	X^2 = 120.8***	X^2 = 8.6**	X^2 = 49.9***
Carrying capacity	X^2 = 534.4***	X^2 = 26.8***	X^2 =1335.2***
Time to fastest growth	X^2 = 448.3***	X^2 = 37.9***	X^2 =1477.8***
Exponential growth rate	X^2 = 384***	X^2 = 29.8***	X^2 = 1410.3***

FIGURE LEGENDS:

Figure 1. Six hypothetical observable outcomes of phenotypic plasticity examined under a phylogenetic framework (a-f). This example portrays any measured phenotypic trait value (e.g. size) measured at three conditions along an environmental gradient (e.g. varied temperature). Phylogenetic signal is visualized along the tips of the phylogenetic tree, with each column representing sizes of four genotypes at a particular temperature. Reaction norms are visualized on each tip of the phylogenetic tree, with each row representing sizes for each genotype across temperatures. Below each phylogeny are reaction norm plots. For each of the six potential outcomes we can infer potential evolutionary and ecological processes for that trait: a) evolution of the trait and of trait plasticity, b) evolution of the trait and a conserved plastic response to temperature, c) evolution of the trait and lack of temperature-dependent plasticity, d) rapid evolution of the trait and highly localized adaptation to temperature, e) rapid evolution of the trait and a conserved response to temperature, f) random change relative to genotype and environment, as might occur through rapid genetic drift. Based on synthesis of Scheiner (1993), Pigliucci et al., (1999), Revell et al., (2008), Matesanz et al., (2010), Nicotra et al., (2010), and Davies et al., (2013).

Figure 2. Reaction norms showing the interactive effect of *Batrachochytrium dendrobatidis* genotype and temperature on (a) zoospore size and (b) zoosporangium size as determined from linear-mixed effects models estimates.

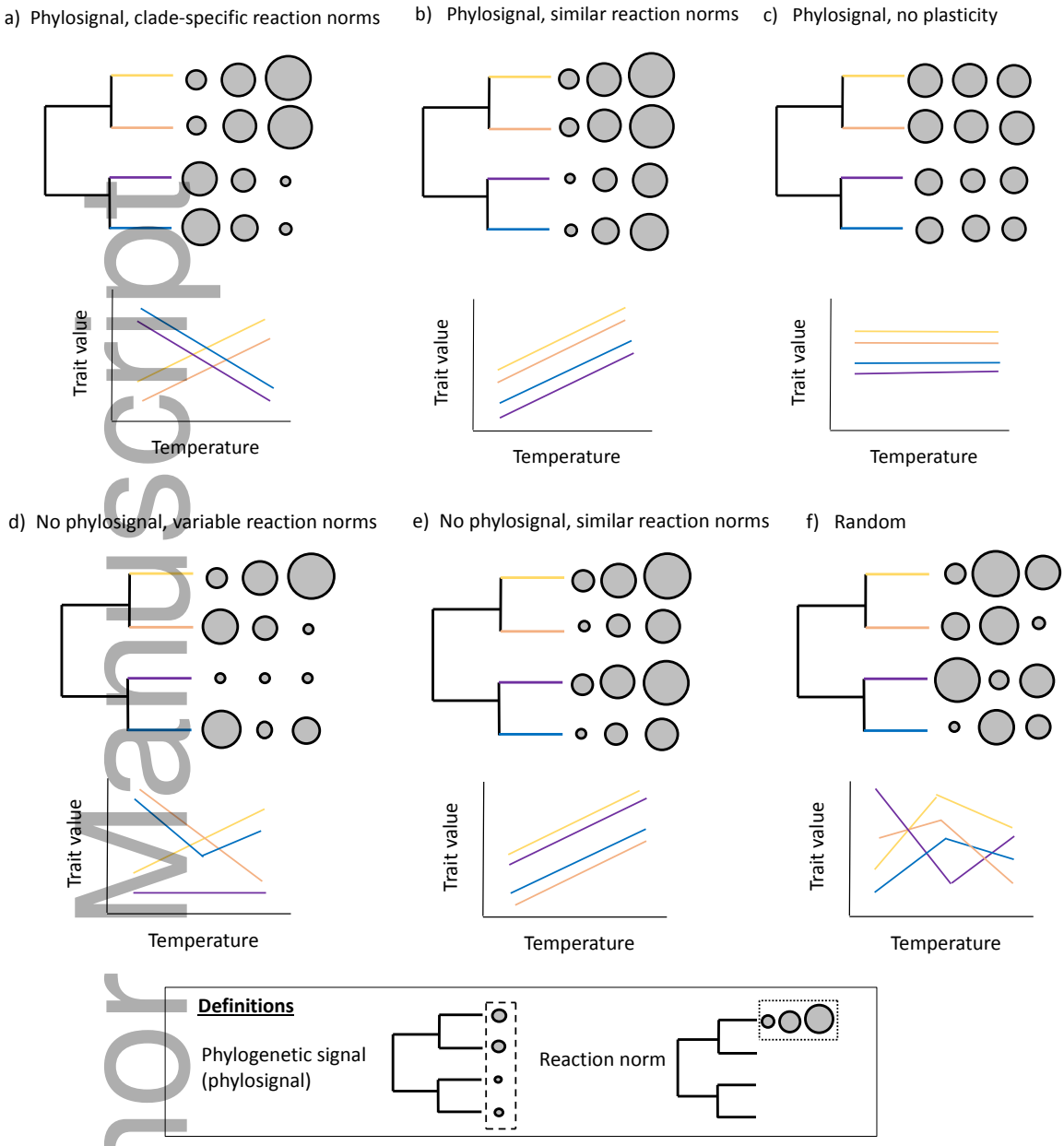
Figure 3. Reaction norms showing the interactive effect of *Batrachochytrium dendrobatidis* genotype and temperature on life history traits a) carrying capacity, b) time to most rapid growth, c) exponential growth rate as determined from logistic growth models and linear-mixed effects model estimates.

Figure 4. Logistic growth models for each genotype by temperature.

Figure 5. Phenotypic plasticity and evolutionary lability in morphological traits. The phylogenetic tree is shown beside the corresponding phenotypic trait values for each Bd genotype. The size of the circle represents the scaled mean trait value for that given trait. The color of the circle represents the quartile of that scaled mean trait value. Yellow circles are trait values below the 25^{th} percentile, green are between the 25^{th} and 75^{th} percentile, and blue are above the 75^{th} percentile. NS indicates that the trait values were not different across temperatures (i.e., no phenotypic plasticity).

Figure 6. Phenotypic plasticity and evolutionary lability in life history traits. The phylogenetic tree is shown beside the corresponding phenotypic trait values for each Bd genotype. The size of the circle represents the scaled mean trait value for that given trait. The color of the circle represents the quartile of that scaled mean trait value. Yellow circles are trait values below the 25^{th} percentile, green are between the 25^{th} and 75^{th} percentile, and blue are above the 75^{th} percentile. NS indicates that the trait values were not different across temperatures (i.e., no phenotypic plasticity).

FIGURES:



780

781 Figure 1.

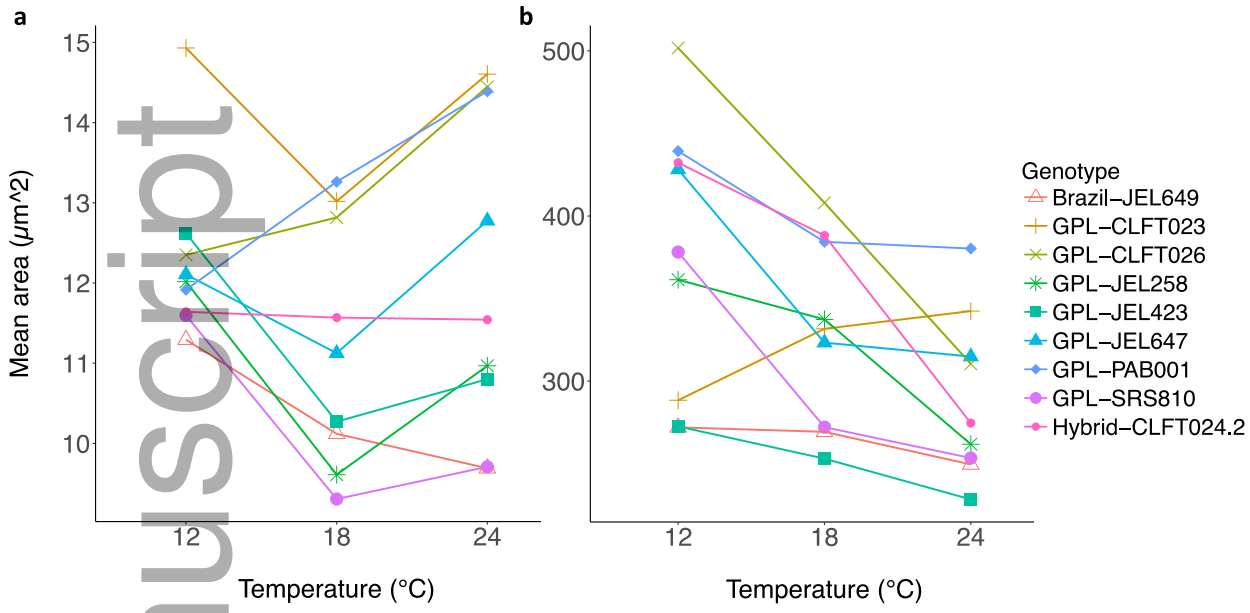
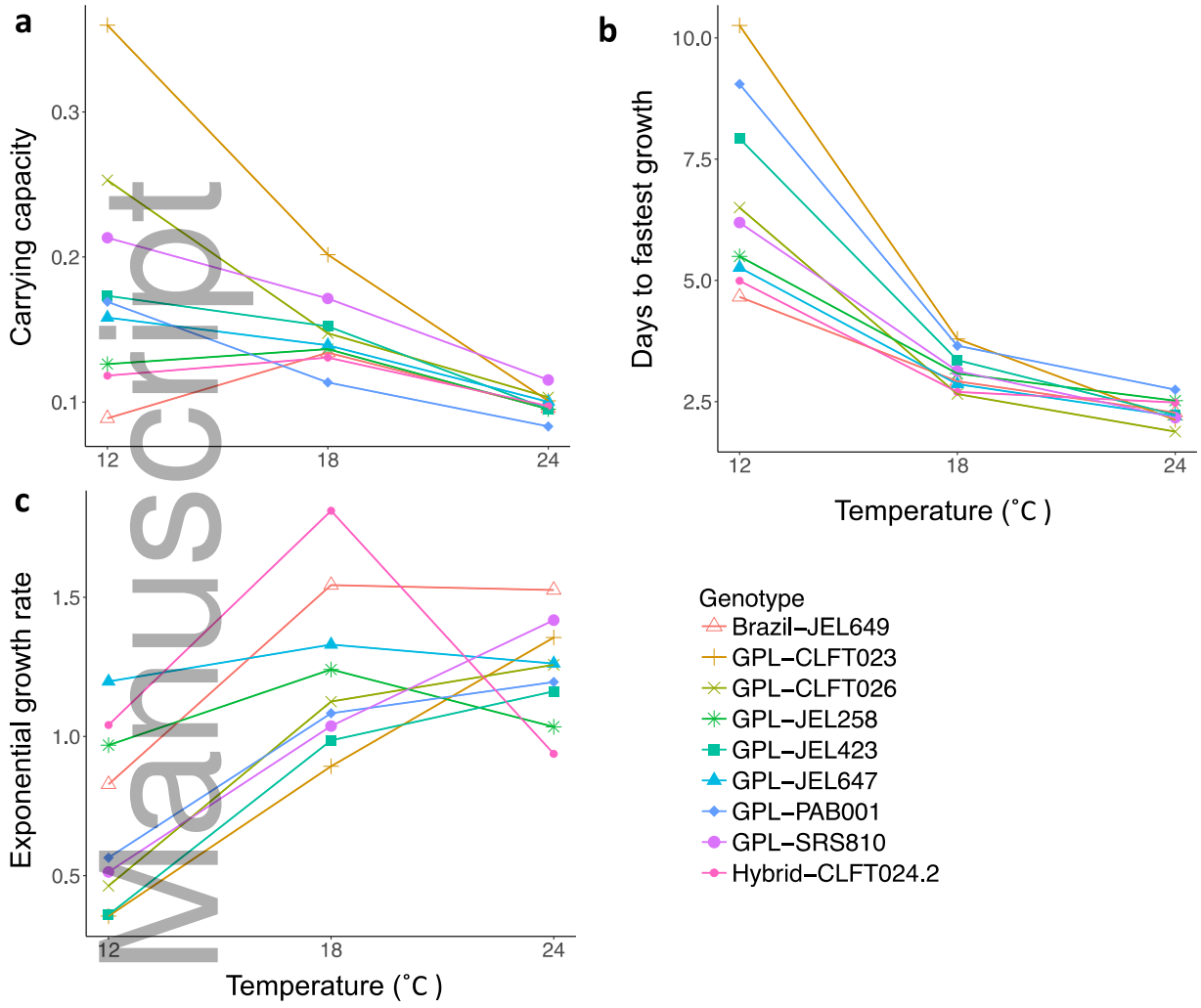


Figure 2.



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787 Figure 3.
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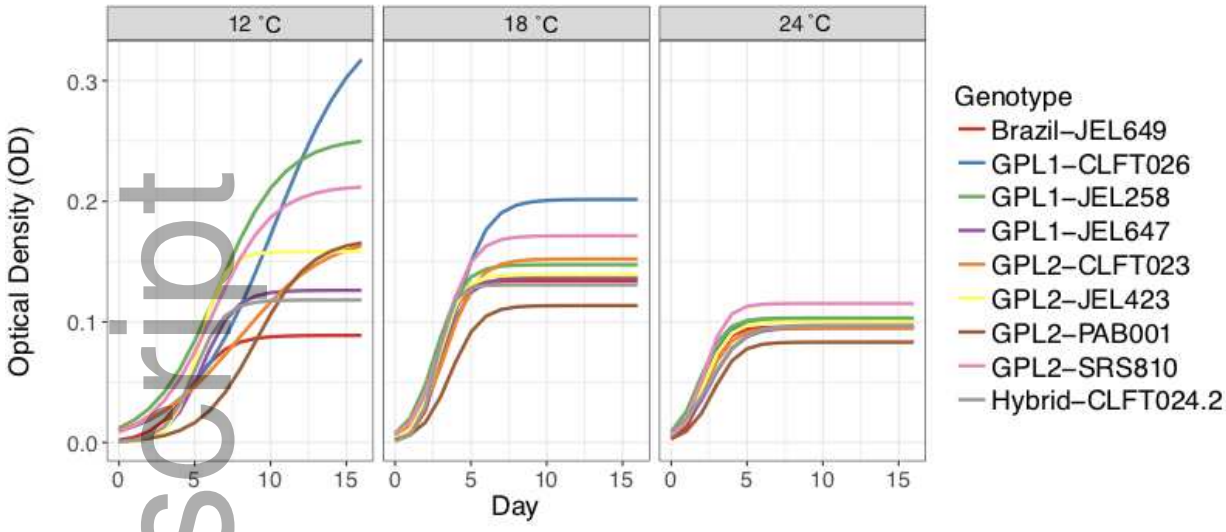

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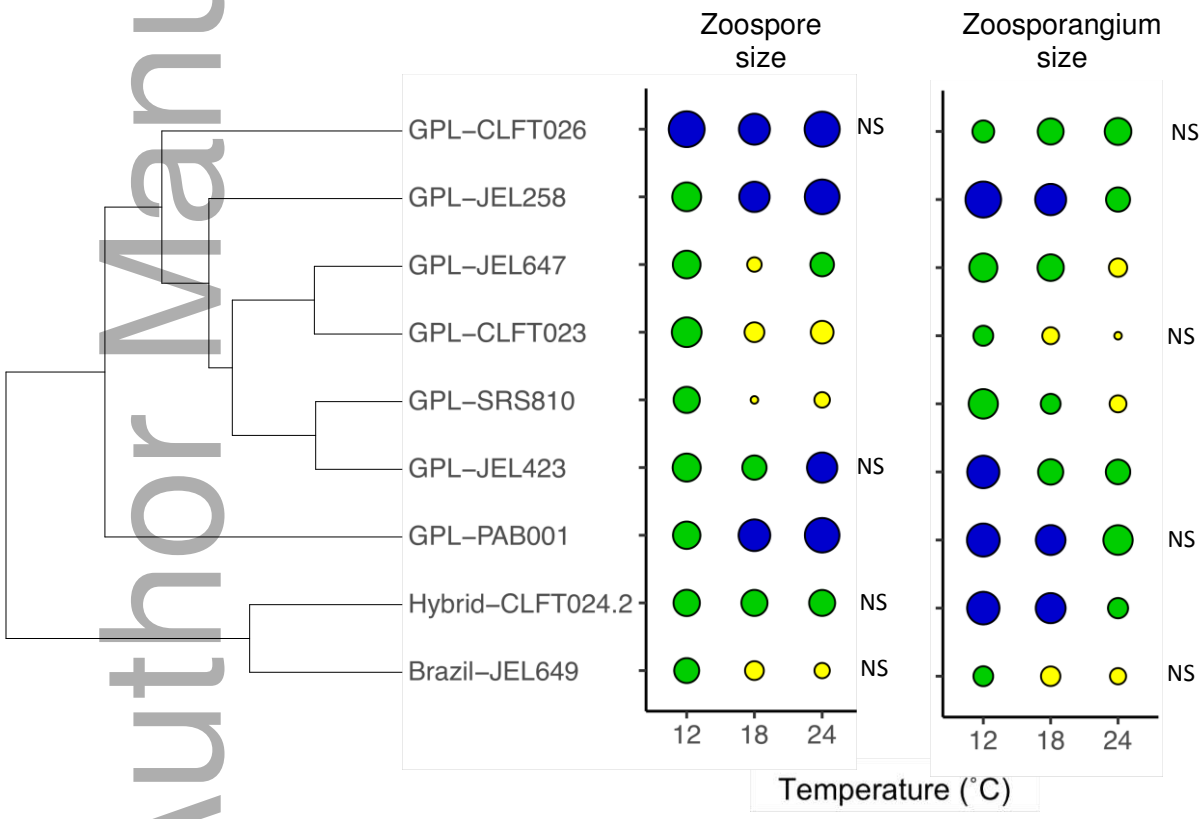
790 Figure 4.



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792 Figure 5.

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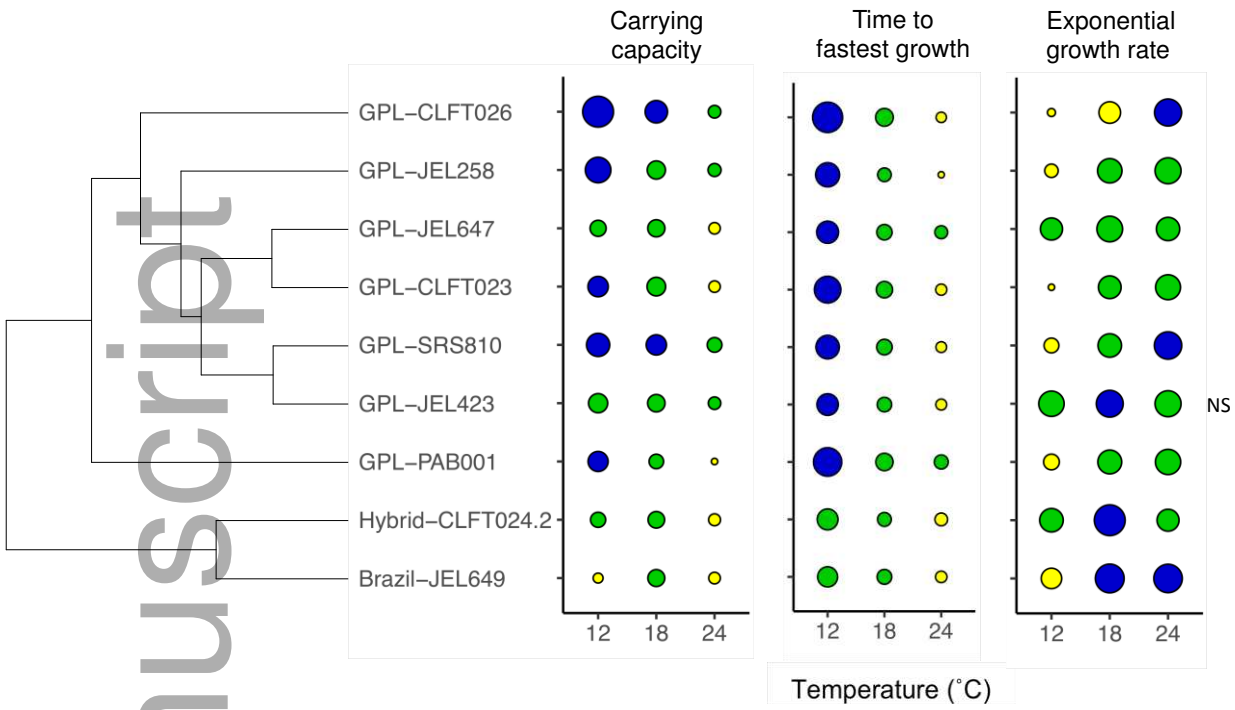

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795 Figure 6.