

1 *Research Manuscript*

2 **Differential thermotolerance adaptation between** 3 **species of *Coccidioides***

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29 Abstract. Coccidioidomycosis, or Valley fever, is caused by two species of dimorphic fungi. Based
30 on molecular phylogenetic evidence, the genus *Coccidioides* contains two reciprocally monophyletic
31 species: *C. immitis* and *C. posadasii*. However, phenotypic variation between species has not been
32 deeply investigated. We therefore explored differences in growth rate under various conditions. A
33 collection of 39 *C. posadasii* and 46 *C. immitis* isolates, representing the full geographical range of the
34 two species, were screened for mycelial growth rate at 37°C and 28°C on solid media. The radial
35 growth rate was measured over 16 days on yeast extract agar. A linear mixed effect model was used
36 to compare the growth rate of *C. posadasii* and *C. immitis* at 37°C and 28°C respectively. *C. posadasii*
37 grew significantly faster at 37°C, when compared to *C. immitis*; whereas both species had similar
38 growth rates at 28°C. These results indicate thermotolerance differs between these two species. As
39 the ecological niche has not been well-described for *Coccidioides* spp., and disease variability
40 between species has not been shown, the evolutionary pressure underlying the adaptation is
41 unclear. However, this research reveals the first significant phenotypic difference between the two
42 species that directly applies to ecological and clinical research.

43 **Keywords:** coccidioidomycosis, fungal pathogen, phenotypic variation, growth rate, Valley fever

44 **1. Introduction**

45 Coccidioidomycosis, or Valley fever, is an environmentally acquired disease caused by
46 inhalation of arthroconidia of dimorphic fungi belonging to the genus *Coccidioides*. In the
47 environment, the fungi grow as filamentous mycelia, alternate cells of which autolyze and become
48 fragile, leaving intact asexual arthroconidia that may disperse via wind or soil disruption. If inhaled

49 by a susceptible host, an arthroconidium switches to a host-associated lifecycle and develops into a
50 specialized infectious structure called a spherule. Subsequently, the host's immune system either
51 represses spherule replication or the host succumbs to the illness (1, 2). It is thought that symptomatic
52 infection occurs in approximately 40% of human patients, who exhibit a broad spectrum of clinical
53 symptoms, ranging from acute self-limited pneumonia, fibrocavitary chronic pulmonary infection,
54 or hematogenous spread to extrapulmonary locations (i.e. disseminated infection) (3). By one
55 estimate, there are 146,000 new symptomatic U.S. coccidioidal infections each year (4) although the
56 reported cases are substantially lower (5).

57 Coccidioidomycosis is caused by two species, *C. immitis* and *C. posadasii*. Genetic analysis of
58 multiple molecular markers has defined two monophyletic clades (6). Subsequent population
59 genetic/genomic studies revealed that *C. immitis* is composed of at least two populations in the
60 western U.S., and *C. posadasii* is composed of three populations widely dispersed across the American
61 continents (7-10). Given the high number of autapomorphic mutations between *Coccidioides* species
62 and among isolates within species, variation in phenotypes is predicted (11). However, minimal work
63 characterizing phenotypic differences has been undertaken. A previous study demonstrated that *C.*
64 *immitis* *in vitro* spherules grew in a synchronous pattern where *C. posadasii* isolates did not (12).
65 Differences in pathogenesis and other disease-associated phenotypic characteristics among strains
66 have been reported, although only one study had species information (13-18). The publication that
67 defined the novel species *C. posadasii* also found species-specific variance in growth rate on media
68 containing 0.136M NaCl, suggesting that *C. immitis* is more salt tolerant than *C. posadasii*, but due to
69 overlap in the phenotype, and evaluation of only 10 isolates of each species, it was not statistically
70 meaningful (6). These data supported observations published in the 1950s - 60s, which proposed that
71 salinity of the soil may be a factor in determining the distribution of *C. immitis* in Californian soil (19-
72 21). In contrast, a correlation of *C. posadasii* with saline soils was not observed in Arizona, where other
73 associations were observed (22-26). Importantly, recent modeling analysis predicts the future
74 expansion of *Coccidioides* species in response to climate dynamics (27). Therefore, a robust
75 investigation of abiotic tolerances that may either limit or enhance distribution of *Coccidioides* is
76 needed (1, 28, 29). Such vital information could provide clues regarding the ecological niche,
77 geographical range limits, or host-specific adaptations of the two species of *Coccidioides*.

78 The division of *Coccidioides* into two species has been challenged by clinicians because of the lack
79 of apparent difference in disease manifestation caused by the two pathogens, but recent work
80 suggests that there might be differences in dissemination patterns between the species (1, 2, 30).
81 Unfortunately, diagnosis and treatment of coccidioidomycosis does not require clinicians to identify
82 to species. The current diagnostic methods; AccuProbe® (31), CocciDx (32), and CocciENV (33), do
83 not distinguish between the two species. Molecular-based technologies exist to differentiate the two
84 species, but these have not been adapted to clinical use (34, 35). However, genotyping the causative
85 agent would allow correlation of clinical presentations and outcomes associated with species. Severe
86 disease and death typically occurs in high risk group patients; however, seemingly healthy
87 individuals can succumb as well, without a known host immunologic or pathogen genotypic
88 explanation (36). Currently, the range of disease manifestations is suggested to be primarily due to
89 host factors (37, 38). There are data supporting variation of virulence among individual isolates, but
90 there is limited research on the subject (1, 13, 16, 17, 39). A reasonable hypothesis would acknowledge
91 that both host and pathogen genetics play a role in disease outcome and should be further
92 investigated (40-43). *Coccidioides*, like other primary fungal pathogens has evolved to withstand 37°C,
93 mammalian body temperature which contributes to establishing host infection (44, 45).

94 This phenomenon, thermotolerance is an intrinsic characteristic of an organism that allows for
95 tolerance of excessively high temperatures. Heat acclimation can shape natural populations for a
96 wide range of microorganisms, and is a physiological adaptation to heat stress imposed by the
97 colonization of new habitats, global climate change and encountering new hosts (46-54). This
98 "preadaptation" is particularly important to pathogenic fungi that tolerate growth in high
99 temperatures, which allows colonization of mammalian tissues (55, 56). For example, *Coccidioides* is
100 adapted to grow at high temperatures in the environment (i.e. North and South American deserts),

101 and is able to colonize a wide range of endothermic hosts throughout the Americas (57-61). *C. immitis*
102 is endemic to the California Central Valley, whereas *C. posadasii* is widely distributed, but has highest
103 prevalence in the Sonoran Desert. The annual mean temperature varies between the hotspot areas,
104 with the California Central Valley having more mild temperatures compared to the Sonoran Desert,
105 which led us to hypothesize that *C. posadasii* is more thermotolerant than *C. immitis*. Therefore, we
106 investigated the growth rate of both species at 37°C representing host temperature and 28°C to
107 support environmental growth conditions, so that we might elucidate species-specific phenotypic
108 variation. Here we demonstrate thermotolerance dissimilarity of the two species by analyzing growth
109 rates of 85 isolates at these two temperatures.

110 2. Materials and Methods

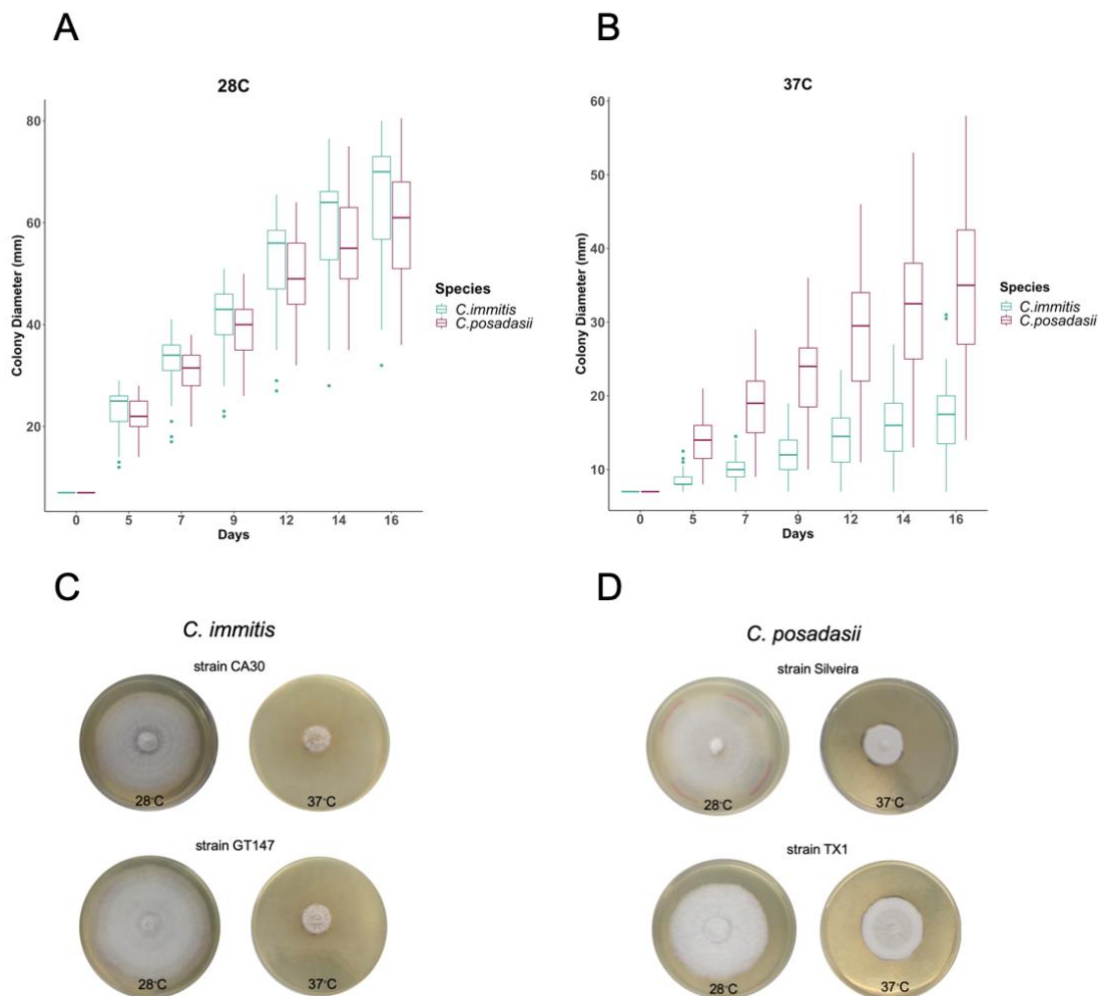
111 Strains and Media. 39 *C. posadasii* strains and 46 *C. immitis* strains used in this study are primarily
112 human patient isolates archived by various institutions, as detailed in Table 1 (6, 8, 28, 62). These
113 strains represent both the full geographic range of the two species, and the proposed geographically
114 distinct sub-populations (6, 8). Strains were grown on 2xGYE media (2% glucose, 1% yeast extract,
115 1.5% agar w/v) to supply initial plugs to inoculate plates for growth analysis. Yeast Extract (YE) media
116 (0.5% yeast extract, 1.5% agar w/v) was used for growth experiments. Flagstaff Medical Center
117 isolates were collected under IRB No. 764034 through Northern Arizona Healthcare as part of the
118 Northern Arizona University Biobank.

119 Growth Conditions and Measurements. Colonies were started by spreading approximately 10⁶
120 arthroconidia over the entire surface of a 2xGYE plate to create a lawn of mycelium to be transferred
121 to initiate the thermotolerance experiment; this allowed measurement of colonial growth and not
122 spore germination differences. After five days of growth at 25°C, 7mm diameter mycelial plugs were
123 subcultured to the center of YE plates using a transfer tool (Transfertube® Disposable Harvesters,
124 Spectrum® Laboratories). Three replicates of each strain were plated for each experiment. All plates
125 (100mm x 15mm BD Falcon 1015) were sealed with gas permeable seals (labtape form TimeMed
126 Labeling Systems, Inc or Key Scientific plate seals) for safety. Plates were placed in temperature-
127 controlled incubators at either 28°C or 37°C in the dark under ambient humidity (30-50% RH) and
128 CO₂ (0.1%) conditions. Plate stacks were rotated from top to bottom and repositioned in the incubator
129 with each measurement timepoint to reduce effects of environmental variation within the incubators.
130 For measurement of radial growth, the diameter of each colony was measured in mm at 5, 7, 9, 12,
131 14, and 16 days post-subculture. The initial experiment proceeded at University of Arizona (UA) and
132 subsequent testing with a new set of isolates occurred at Northern Arizona University (NAU). Details
133 for strains tested at each institution are listed in Table 1 and all raw measurement data are available
134 in File S1.

135 Statistical Analysis. To estimate the mean growth rate for each species over the two-week period
136 a mixed effect linear model for each temperature was constructed using the lme4 package in R version
137 3.6.2 (63, 64). Initially, data sets were divided by institution and after concluding that species specific
138 growth rate was not impacted by collection site the data sets were combined (Table S1). In the
139 temperature specific models, the factors “day” and “species” were assumed to be fixed linear effects,
140 and individual isolate response for each day was considered to be a normally distributed random
141 effect as appropriate in a longitudinal study. Thus, the response variable of colony diameter was
142 modeled with fixed effects and a random effect to determine if growth rates varied between strains
143 at either 28°C and 37°C. Shapiro-Wilk test (p-value < 0.001) shows that residuals are not normally
144 distributed. However, the large sample size and overall residual structure support that a linear model
145 is the most appropriate for this data set. In addition, bootstrapping using the boot package in R (65,
146 66) was used to estimate 95% confidence intervals (CIs) for growth rates and other fixed effects
147 (nsim=2,000). All bootstrap parameters were similar and support model estimates. A comparison
148 between bootstrapped CIs and CIs constructed using the linear model can be found in S1 Table and
149 S2 Table.

150 3. Results

151 To define variability of one phenotypic trait between two *Coccidioides* species, we examined the
 152 ability of *Coccidioides* spp. to grow in filamentous form at 37°C and 28°C on yeast extract (YE) agar. In
 153 this study, we surveyed 85 strains of *Coccidioides*, representing isolates from the entire geographical
 154 range of *Coccidioides*, for growth rate differences between species at 37°C and 28°C (Figure 1). Initial
 155 investigations occurred at the University of Arizona, and subsequent studies occurred at Northern
 156 Arizona University (Table 1).



157

158

Table 1. Strain information

ID	Species	Geographical Origin ^a	Source	Testing Institution
CA22	<i>C. immitis</i>	California	University of Texas Health Science Center (UTHSC)	NAU
500	<i>C. posadasii</i>	Soil, Tucson, AZ	University of Arizona (UA)	UA
IL1	<i>C. posadasii</i>	Illinois	UTHSC	NAU
CA23	<i>C. immitis</i>	California	UTHSC	NAU
HS-I-000718	<i>C. posadasii</i>	Arizona	Flagstaff Medical Center (FMC)	NAU
GT164	<i>C. posadasii</i>	Texas	University of California Davis (UCD)	NAU
GT163	<i>C. immitis</i>	California	UCD	NAU
HS-I-000588	<i>C. posadasii</i>	Arizona	FMC	NAU
CA28	<i>C. immitis</i>	California	UTHSC	NAU
TX4	<i>C. posadasii</i>	Texas	UTHSC	NAU

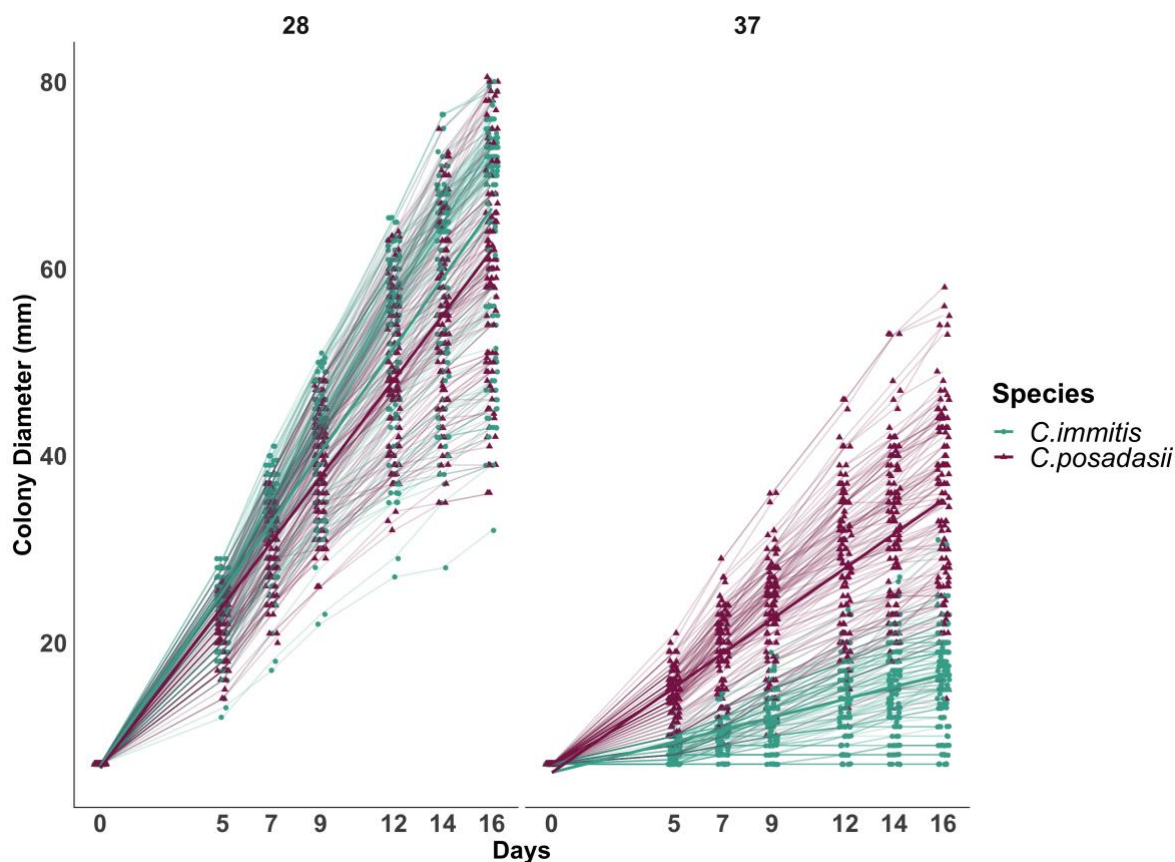
HS-I-000235	<i>C. posadasii</i>	Arizona	FMC	NAU
TX1	<i>C. posadasii</i>	Texas	UTHSC	NAU
HS-I-000778	<i>C. posadasii</i>	Arizona	FMC	NAU
GT147	<i>C. immitis</i>	California	UCD	NAU
HS-I-000234	<i>C. posadasii</i>	Texas	FMC	NAU
CA30	<i>C. immitis</i>	California	UTHSC	NAU
HS-I-000547	<i>C. posadasii</i>	Arizona	FMC	NAU
HS-I-000233	<i>C. posadasii</i>	Arizona	FMC	NAU
GT166	<i>C. posadasii</i>	Texas	UCD	NAU
CA24	<i>C. immitis</i>	California	UTHSC	NAU
CA29	<i>C. immitis</i>	California	UTHSC	NAU
M211	<i>C. posadasii</i>	Central Mexico	Unidad de Micología, UNAM	NAU
GT158	<i>C. posadasii</i>	Arizona	UCD	NAU
CA15	<i>C. immitis</i>	California	UTHSC	NAU
CA27	<i>C. immitis</i>	California	UTHSC	NAU
TX3	<i>C. posadasii</i>	Texas	UTHSC	NAU
CA20	<i>C. immitis</i>	California	UTHSC	NAU
RS	<i>C. immitis</i>	California	Common Laboratory Strain	NAU
Silveira	<i>C. posadasii</i>	California	Common Laboratory Strain	NAU
RMSCC2378	<i>C. posadasii</i>	Argentina	R. Negroni	UA
RMSCC2377	<i>C. posadasii</i>	Argentina	R. Negroni	UA
RMSCC2379	<i>C. posadasii</i>	Argentina	R. Negroni	UA
RMSCC3698	<i>C. immitis</i>	Barstow, California	Naval Hospital	UA
RMSCC3490	<i>C. posadasii</i>	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3505	<i>C. immitis</i>	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3506	<i>C. posadasii</i>	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3472	<i>C. posadasii</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3474	<i>C. immitis</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3475	<i>C. immitis</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3476	<i>C. immitis</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3478	<i>C. posadasii</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3479	<i>C. immitis</i>	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3377	<i>C. immitis</i>	Monterey, California	UCD	UA
RMSCC2343	<i>C. posadasii</i>	Nuevo Leon, Mexico	R. Diaz	UA
RMSCC2346	<i>C. posadasii</i>	Nuevo Leon, Mexico	R. Diaz	UA
RMSCC3738	<i>C. posadasii</i>	Piaui, Brazil	B. Wanke	UA
RMSCC3740	<i>C. posadasii</i>	Piaui, Brazil	B. Wanke	UA
RMSCC2127	<i>C. posadasii</i>	Texas	UTHSC	UA
RMSCC2133	<i>C. posadasii</i>	Texas	UTHSC	UA
RMSCC2234	<i>C. posadasii</i>	Texas	UTHSC	UA
RMSCC2102	<i>C. immitis</i>	San Diego, California	University of California San Diego (UCSD) Medical Center	UA
RMSCC2394	<i>C. immitis</i>	San Diego, California	UCSD Medical Center	UA
RMSCC2395	<i>C. immitis</i>	San Diego, California	UCSD Medical Center	UA

RMSCC3693	<i>C. immitis</i>	San Diego, California	Naval Hospital	UA
RMSCC3703	<i>C. immitis</i>	San Diego, California	UCSD Medical Center	UA
RMSCC3705	<i>C. immitis</i>	San Diego, California	UCSD Medical Center	UA
RMSCC3706	<i>C. immitis</i>	San Diego, California	UCSD Medical Center	UA
RMSCC2006	<i>C. immitis</i>	San Joaquin Valley	Kern County Public Health (KCPH)	UA
RMSCC2009	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2010	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA and NAU
RMSCC2011	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2012	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2014	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2015	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2017	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2268	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2269	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2271	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2273	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2274	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2275	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2276	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2277	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2278	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2279	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2280	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC2281	<i>C. immitis</i>	San Joaquin Valley	KCPH	UA
RMSCC3480	<i>C. posadasii</i>	Sonora, Mexico	I. Gutierrez	UA
RMSCC3487	<i>C. posadasii</i>	Sonora, Mexico	I. Gutierrez	UA
RMSCC3488	<i>C. posadasii</i>	Sonora, Mexico	I. Gutierrez	UA
RMSCC1040	<i>C. posadasii</i>	Tucson, Arizona	UA	UA
RMSCC1043	<i>C. posadasii</i>	Tucson, Arizona	UA	UA
RMSCC1044	<i>C. posadasii</i>	Tucson, Arizona	UA	UA
RMSCC1045	<i>C. posadasii</i>	Tucson, Arizona	UA	UA
RMSCC3796	<i>C. posadasii</i>	Venezuela	G. San-Blas	

159 ^aOften patient diagnosis location

160 We observed that mean growth rates varied slightly between institutions however overall
 161 species-specific temperature behavior remained. Therefore, data sets were combined (Figure S1,
 162 Table S1). Using a mixed effect linear model, we showed a significant species-specific difference for
 163 growth of the mycelial phase of the fungus based on temperature (Figure 2 and Table 2). Table 2
 164 summarizes the estimated growth rate for each species, 95% confidence interval (CI), and p-value for
 165 each temperature specific model. Both species grew quicker at 28°C than 37°C. Although, *C. posadasii*
 166 had a larger mean diameter on all days tested (Table S3) the overall rate of increase was not
 167 statistically significant (p-value = 0.072, Table 2). This was in contrast to growth at 37°C. At this
 168 temperature, *C. posadasii* strains exhibited larger mean diameters, which reached double the diameter
 169 of *C. immitis* by day 16 (Table S3). At this temperature the overall growth rate of *C. posadasii* was
 170 1mm/day faster than *C. immitis* (Figure 2 and Table 2). This difference was statistically significant (p-
 171 value < 0.001, Table 2). These findings were consistent for all days tested, and represent differential
 172 phenotypes for both species. Thus, our analysis indicates that high temperature is the important

173 variable between species growth rate on solid media. This phenotypic difference supports the
 174 molecular phylogenetic species designation and may reflect adaptation of *C. immitis* to cooler
 175 environments, or possibly specific hosts.



176

177 Table 2. Temperature Specific Linear Model Slope Estimates for Radial Growth Rate at 28°C or 37°C.

Species	Colony Diameter at 28°C			Colony Diameter at 37°C		
	mm/day	95% CI	p^a	mm/day	95% CI	p^a
<i>C. immitis</i> x Day	3.73	3.53 – 3.92	0.072	0.64	0.51 – 0.78	<0.001
<i>C. posadasii</i> x Day	3.47	0.55 – 0.02		1.82	0.98 – 1.38	
N ^b	85			85		

^a difference between estimated slope ^b number of isolates

178 Summary of temperature specific linear models, for 28°C and 37°C, respectively. Colony growth estimates for
 179 each species per day (slope), 95% confidence intervals (CI) and p values. At 28°C, *C. immitis* grows 3.73 mm/day
 180 which is 0.26 mm faster per day than *C. posadasii*. The difference in slope is not significant ($p=0.072$) based on $\alpha=$
 181 0.05. However, the p-value trends towards significance. At 37°C, *C. immitis* grows 0.64mm/day which is 1.18mm
 182 slower than *C. posadasii*. The difference in slope (CI, 0.98-1.38 mm/day) is statistically significant ($p<0.001$).

183 3.1 Figures, Tables and Schemes

184 Fig 1. Temperature impacts growth ability of *C. immitis* isolates compared to *C. posadasii* on YE
 185 media. Seven mm diameter plugs were sub-cultured onto yeast extract plates and radial growth was
 186 documented over 16 days. (A) Radial growth measurements at 37°C for 46 *C. posadasii* and 39 *C.*
 187 *immitis* isolates in triplicate. (B) Radial growth measurements at 28°C for 46 *C. posadasii* and 39 *C.*

188 *immitis* isolates in triplicate. (C) Representative samples of phenotypic variation observed between
189 species on day 16.

190 Fig 2. Radial growth rate of 85 isolates of *Coccidioides* demonstrates species-specific response to
191 temperature. Each line represents the mean diameter (y-axis) for each isolate in triplicate (46 *C.*
192 *immitis* and 39 *C. posadasii*) at a given time point (x-axis). Dark lines represent mean growth rate of
193 each species. Radial growth was measured at day 5, 7, 9, 12, 14 and 16. There is a significant difference
194 in growth rate (slope) in response to higher temperature between species of *Coccidioides*. The radial
195 growth rate of *C. immitis* is decreased at a higher temperature 37°C (slope₃₇ = 0.64 mm/day; 95% C.I.
196 0.51-0.78) compared to *C. posadasii* (slope₃₇ = 1.82 mm/day; 95% C.I. 1.49-2.16). Both species appear
197 to tolerate 28°C and grow at a similar rate (*C. immitis* slope₂₈ = 3.73 mm/day; 95% C.I. 3.53-3.92, *C.*
198 *posadasii*, slope₂₈ = 3.47 mm/day; 95% C.I. 2.98-3.90).

199 4. Discussion

200 Although many studies have looked at genetic variation among isolates of both species of
201 *Coccidioides*, few studies have compared phenotypic differences. Observed genetic diversity between
202 and within species makes it reasonable to hypothesize that phenotypic variation exists. We propose
203 that a methodical documentation of phenotypic variation is a necessary first step to determine the
204 ecological or clinical relevance of these traits. In this study, we have identified a definitive phenotypic
205 difference with a congruent analysis at two institutions for a diverse set of isolates. A total of 85
206 isolates covering the geographic range of both species show that *C. posadasii* isolates grow at a
207 significantly faster rate ($p < 0.001$, Fig 2 and Table 2) than *C. immitis* isolates in the mycelial form at
208 37°C on YE agar. Additionally, *C. immitis* grows slightly faster than *C. posadasii* at 28°C on YE agar
209 although the difference in growth rate is not significant (p -value = 0.072, Fig 2 and Table 2). We note
210 that growth rate may be influenced by nutrition source, and the results are limited to the media
211 utilized for the current study.

212 Functionally, this phenotype is similar to a classic temperature sensitive (ts) conditional mutant,
213 such that *C. immitis* exhibits normal growth at permissive temperature, and significantly slower
214 growth under stressful conditions. It is possible that *C. immitis* could be restored to normal growth at
215 37°C by gene replacement with appropriate *C. posadasii* alleles if candidate genes were identified.
216 Several genes and pathways have been described in *Aspergillus fumigatus* related to thermotolerance
217 (54). For example, the observed phenotype could be due to mutations in a heat shock protein (Hsp).
218 Hsps are activated in response to changes in temperature and regulate cellular processes associated
219 with morphogenesis, antifungal resistance, and virulence by triggering a wide array of cellular
220 signaling pathways (53, 67). Hsps are activated by a heat shock transcription factor (Hsf) that acts as
221 a thermosensor, regulating the Hsps at specific growth temperatures (68). Several studies have shown
222 that *Coccidioides* up-regulates heat shock proteins Hsp20 and Hsp9/12 at high temperature during the
223 parasitic lifecycle while down-regulating Hsp30 and Hsp90 (69-72). Further investigation of Hsps
224 and Hsfs in *Coccidioides* could elucidate mechanisms of the species-specific thermotolerant behavior
225 observed in this study. Alternatively, many classical ts mutants occur in genes required for normal
226 cellular growth and are due to single amino acid changes that affect protein function or stability at
227 the restrictive temperature. For example, a number of colonial temperature sensitive (*cot*) mutants
228 have been identified in *Neurospora crassa*. The *N. crassa cot-1* mutant has been studied in greatest
229 detail, and the ts defect is due to a SNP causing a single amino acid change in a Ser/Thr protein kinase
230 required for normal hyphal extension, thus resulting in restricted growth at normally permissive
231 temperatures above 32°C (73, 74). Finally, recent work in *Saccharomyces* indicates that mitochondrial
232 genotypes are associated with heat tolerance (75). The mitochondrial genomes of the two species of
233 *Coccidioides* are also distinct, and thus mitochondrial function is another potential mechanism
234 controlling thermotolerance in *Coccidioides*.

235 The source of the genotypic variation driving the observed phenotype may be attributable to a
236 stochastic event, such as a founder effect or population bottleneck 10-12 MYA, which is the estimated
237 time the two species have been separated (6, 76). Alternatively, the observed pattern may be due to
238 selection pressure from a specific environment, host, or directly associated with virulence. Thus, the

239 observed differential thermotolerance may relate to the saprobic phase of the lifecycle and reflect
240 adaptation to specific environments. A pattern of alternating wet-dry conditions has been related to
241 Valley fever incidence across the southwestern U.S. (5, 77-81). It has been proposed that fungal
242 growth occurs during brief periods of heavy moisture during monsoon and winter rainy seasons in
243 the Southwest, which are followed by prolific conidia production when warm temperatures and low
244 rainfall desiccate soils and increase dispersal via dust (the “grow and blow” hypothesis) (27, 78, 82).
245 Additionally, during high temperature periods, it is hypothesized that the surface soil is partially
246 sterilized and many competitors are removed, but *Coccidioides* spores remain viable (26). Another
247 hypothesis is that *C. posadasii* may be better adapted to growth in the high soil temperatures observed
248 in the southwestern deserts compared to the California endemic *C. immitis*. Maricopa, Pinal and Pima
249 counties harbor the highest coccidioidomycosis case rates in Arizona due to *C. posadasii*, and
250 according to the National Centers for Environmental Information (83), the annual mean temperature
251 (1901-2000) were 20.7°C, 19.8°C and 19.2°C, respectively. On the other hand, Fresno, King and Kern
252 counties, which harbor the highest coccidioidomycosis case rates in California due to *C. immitis*, had
253 annual mean temperatures of 12.4°C, 16.9°C and 15.8°C, respectively. The difference in 100-year
254 average annual mean temperature between highly endemic areas of Arizona and California supports
255 our hypothesis that *C. posadasii* is more adapted to higher temperatures compared to *C. immitis*.
256 Alternatively, a preferred host species may vary in normal body temperature, in accordance with the
257 endozoan small mammal reservoir hypothesis proposed by Barker and Taylor (84). Interestingly, a
258 decline in mean human body temperature (~1.6%) has recently been reported (85). Whether this
259 impacts coccidioidomycosis rates is unknown.

260 Published literature to date suggests that disease outcomes are related primarily to host-specific
261 factors (37, 38, 86), and certainly, host genetic background can impact disease progression. We
262 propose that pathogen-specific variation may also contribute to capricious disease outcomes in
263 coccidioidomycosis patients. Currently, species-specific virulence is not well-documented in
264 *Coccidioides* research, but has been suggested (1, 87). This is in part due to the use of a few
265 characterized laboratory strains of *Coccidioides* for most hypothesis testing, primarily strains Silveira,
266 C735 and RS (70, 86, 88-91). Therefore, connecting phenotypic dissimilarity to established genetic
267 variation using genome-wide association studies could provide insight into unique characteristics of
268 these genetically distinct pathogens.

269 5. Conclusions

270 In summary, we have identified a significant phenotypic difference between *C. immitis* and *C.*
271 *posadasii*. Although growth rate on YE media at two temperatures is the only characteristic we
272 explicitly tested, there are certain to be more phenotypic differences between species, and possibly
273 between populations. This, coupled with the recent availability of the genome sequence of multiple
274 strains for both fungal species, may allow comparative genomic approaches to elucidate candidate
275 genes for thermotolerance regulation in *Coccidioides* and closely related Onygenales (7).

276 Supplementary Materials:

277 Fig S1. Growth of *C. immitis* and *C. posadasii* on YE media at NAU and UA. Seven mm diameter plugs were sub-
278 cultured onto yeast extract plates and radial growth was documented over sixteen days. (A) Radial growth
279 measurements at 28°C and 37°C for 85 isolates in triplicate, at both institutions. (B) Representative samples of
280 phenotypic variation observed between species on day sixteen for both NAU and UA experiments.

281 Table S1. Analysis of variance. Impact of institution collection site was investigated for each temperature specific
282 model. The factors “day”, “species”, and “lab” were assumed to be fixed linear effects, and individual isolate
283 response for each day was considered to be a normally distributed random effect as appropriate in a longitudinal
284 study. The Factor “Lab” location adds variation to the data sets but does not alter overall findings. Species
285 specific behavior based on temperature pattern remain.

286

287 Table S2. Mean Colony Diameter at 28°C. Mean diameter and standard deviation for *C. posadasii* and *C. immitis*
288 at 28°C. Welch's t-test was used to compare difference in means. Mean is significantly different on all days tested.
289 Significance is reduced on day 16.

290 Table S3. Mean Colony Diameter at 37°C. Mean diameter and standard deviation for *C. posadasii* and *C. immitis*
291 at 37°C. Welch's t-test was used to compare difference in means at each time point. Mean is significantly different
292 on all days tested.

293 Table S4 Table. Comparison Linear Model and Bootstrap Values. Comparison of linear model and bootstrap 95%
294 confidence intervals for 28°C and 37°C data sets. Bootstrapping conducted using the boot package in R.

295 S1 File. Final Raw Data for Temperature Differences at 37 °C and 28 °C. Measurements (diameter in mm) for
296 each isolate on each plate were recorded on days 5, 7, 9, 12, 14, and 16. Three replicates were completed for each
297 strain for both temperature conditions. Strain details are listed in Table 1.

298 **Author Contributions:**

299 H.L.M., P.L.H., M.M.T., C.S.W., M.J.O. and B.M.B. prepared the initial draft of the manuscript. B.M.B., M.J.O.,
300 and J.N.G. developed the concept, provided funding, and were responsible for approving the final draft of the
301 manuscript. M.M.T., H.L.M., P.L.H. assisted with creation of figures and writing final manuscript. I.N.S, H.L.M.,
302 and C.S.W. developed statistical models. H.L.M., P.L.H., B.M.B., performed experiments and collected data.
303 N.P.W, G.R.T. III, R.M-S, L.R.C-O, P.K., C.P., J.T., J.N.G., provided isolates. All authors have read and agreed
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319 **Conflicts of Interest:**

320 The authors declare no conflict of interest.

321

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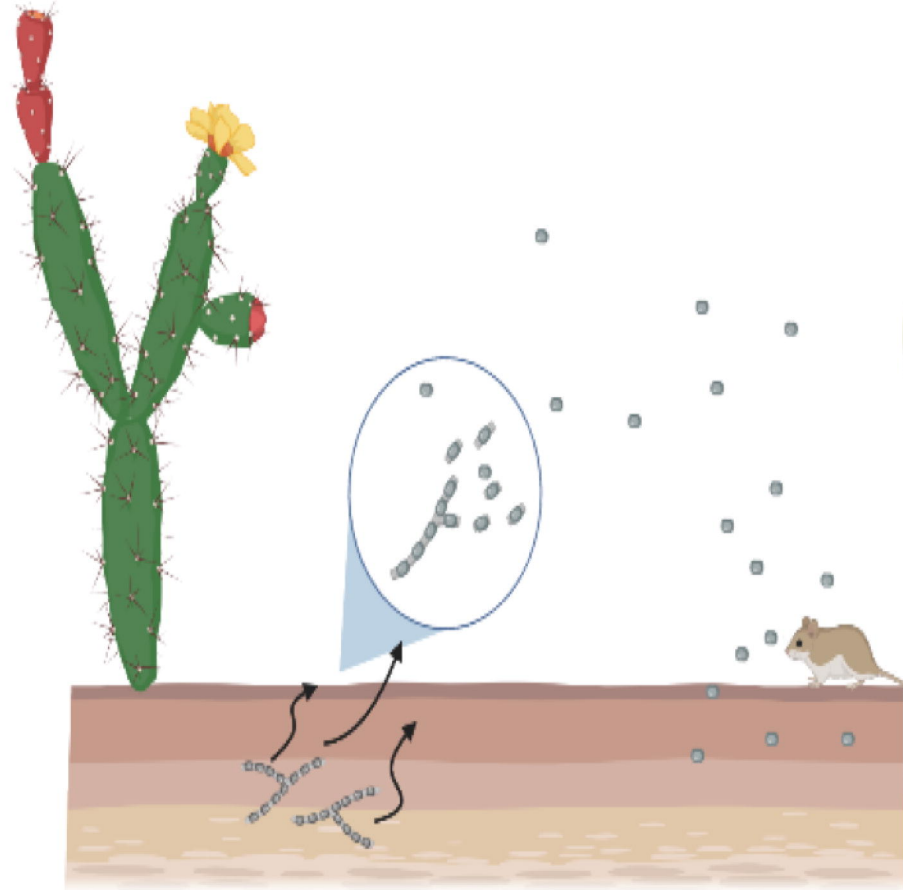
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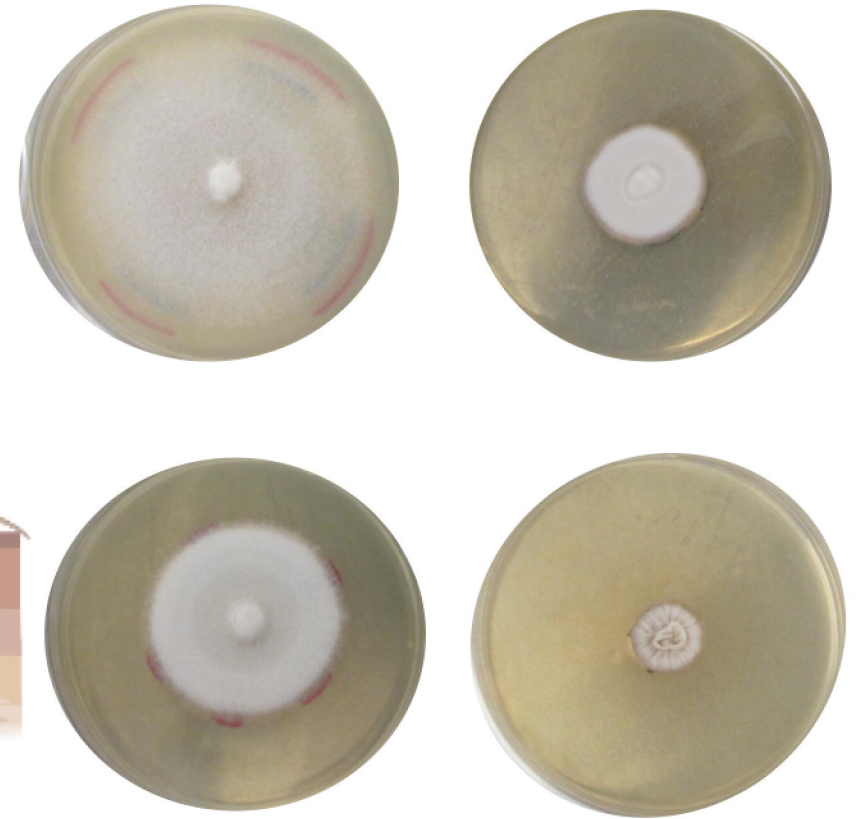
C. posadasii

C. immitis



28°C

37°C



Onygenaceae or dimorphic fungi