Differential thermotolerance adaptation between species of *Coccidioides*

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Abstract

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

Author Summary

The two species of *Coccidioides* are genetically distinct. However, phenotypic variation has not been well-characterized. In this study we identify a significant and reproducible phenotypic difference between the two species, namely that *C. posadasii* grows faster at 37°C than *C. immitis* on yeast extract agar. This is the first significant phenotypic difference

documented for multiple strains across the geographic range of *Coccidioides*. The clinical or ecological relevance of this observation remains to be elucidated.

Introduction

Coccidioidomycosis, or Valley fever, is an environmentally acquired disease caused by inhalation of arthroconidia of dimorphic fungi belonging to the genus *Coccidioides*. In the environment, the fungi grow as filamentous mycelia, alternate cells of which autolyze and become fragile, leaving intact asexual arthroconidia that may disperse via wind or soil disruption. If inhaled by a susceptible host, an arthroconidium switches to a host-associated lifecycle and develops into a specialized infectious structure called a spherule. Subsequently, the host's immune system either represses spherule replication or the host succumbs to the illness [1, 2]. It is thought that symptomatic infection occurs in approximately 40% of human patients, who exhibit a broad spectrum of clinical symptoms, ranging from acute self-limited pneumonia, fibrocavitary chronic pulmonary infection, or hematogenous spread to extrapulmonary locations (disseminated infection) [3]. By one estimate, there are 146,000 new symptomatic U.S. coccidioidal infections each year [4].

Coccidioidomycosis is caused by two species, *C. immitis* and *C. posadasii*. Genetic analysis of multiple molecular markers has defined two monophyletic clades [5]. Subsequent population genetic/genomic studies revealed that *C. immitis* is composed of at least two populations in the western U.S., and *C. posadasii* is composed of three populations widely dispersed across the American continents [6-9]. Given the high number of autapomorphic mutations between *Coccidioides* species and among isolates within species, variation in phenotypes is predicted [10]. However, minimal work characterizing phenotypic differences

77 has been undertaken. A previous study demonstrated that *C. immitis in vitro* spherules grew
78 in a synchronous pattern where *C. posadasii* isolates did not [11]. Differences in pathogenesis
79 and other disease-associated phenotypic characteristics among strains have been reported,
80 although only one study had species information [12-17]. The publication that defined the
81 novel species *C. posadasii* also found species-specific variance in growth rate on media
82 containing 0.136M NaCl, suggesting that *C. immitis* is more salt tolerant than *C. posadasii*,
83 but due to overlap in the phenotype, and evaluation of only 10 isolates of each species, it was
84 not statistically meaningful [5]. These data supported observations published in the 1950s -
85 60s, which proposed that salinity of the soil may be a factor in determining the distribution of
86 *C. immitis* in Californian soil [18-20]. In contrast, a correlation of *C. posadasii* with saline
87 soils was not observed in Arizona, where other associations were observed [21-25].
88 Importantly, recent modeling analysis predicts the future expansion of *Coccidioides* species
89 in response to climate dynamics [26]. Therefore, a robust investigation of abiotic tolerances
90 that may either limit or enhance distribution of *Coccidioides* is needed [1, 27, 28]. Such vital
91 information could provide clues regarding the ecological niche, geographical range limits, or
92 host-specific adaptations of the two species of *Coccidioides*.

93 The division of *Coccidioides* into two species has been challenged by clinicians
94 because of the lack of apparent difference in disease manifestation caused by the two
95 pathogens, but recent work suggests that there might be differences in dissemination patterns
96 between the species [1, 2, 29]. Unfortunately, diagnosis and treatment of coccidioidomycosis
97 does not require clinicians to identify to species. The current diagnostic methods; AccuProbe®
98 [30], CocciDx [31], and CocciENV [32], do not distinguish between the two species.
99 Molecular-based technologies exist to differentiate the two species, but these have not been

adapted to clinical use [33, 34]. However, genotyping the causative agent would allow correlation of clinical presentations and outcomes associated with species. Severe disease and death typically occurs in high risk group patients; however, seemingly healthy individuals can succumb as well, without a known host immunologic or pathogen genotypic explanation [35]. Currently, the range of disease manifestations is suggested to be primarily due to host factors [36, 37]. There are data supporting variation of virulence among individual isolates, but there is limited research on the subject [1, 12, 15, 16, 38]. A reasonable hypothesis would acknowledge that both host and pathogen genetics play a role in disease outcome [39-42].

Thermotolerance is an intrinsic characteristic of an organism that allows for tolerance of excessively high temperatures. Heat acclimation can shape natural populations for a wide range of microorganisms, and is a physiological adaptation to heat stress imposed by the colonization of new habitats, global climate change and encountering new hosts [43-51]. This "preadaptation" is particularly important to pathogenic fungi that tolerate growth in high temperatures, which allows colonization of mammalian tissues [52, 53]. For example, *Coccidioides* is adapted to grow at high temperatures in the environment (i.e. North and South American deserts), and is able to colonize a wide range of endothermic hosts throughout the Americas [54-58]. *C. immitis* is endemic to the California Central Valley, whereas *C. posadasii* is widely distributed, but has highest prevalence in the Sonoran Desert. The annual mean temperature varies between the hotspot areas, with the California Central Valley having more mild temperatures compared to the Sonoran Desert, which led us to hypothesize that *C. posadasii* is more thermotolerant than *C. immitis*. Therefore, we investigated the growth rate of both species at 37ºC and 28ºC, so that we might elucidate species-specific phenotypic

variation. Here we demonstrate thermotolerance dissimilarity of the two species by analyzing growth rates of 85 isolates at these two temperatures.

Results

To define variability of one phenotypic trait between two *Coccidioides* species, we examined the ability of *Coccidioides* spp. to grow in filamentous form at 37ºC and 28ºC on yeast extract (YE) agar. Growth rate differences were observed between *C. immitis* and *C. posadasii*, with the growth of *C. immitis* significantly reduced ($p<0.001$) at 37ºC compared to *C. posadasii* (Fig 1). In contrast, both strains grew equally well at 28ºC (p-value = 0.072).

Fig 1. Temperature impacts growth ability of *C. immitis* isolates compared to *C. posadasii* on YE media. Seven mm diameter plugs were sub-cultured onto yeast extract plates and radial growth was documented over 16 days. (A) Radial growth measurements at 37ºC for 46 *C. posadasii* and 39 *C. immitis* isolates in triplicate. (B) Radial growth measurements at 28ºC for 46 *C. posadasii* and 39 *C. immitis* isolates in triplicate. (C) Representative samples of phenotypic variation observed between species on day 16.

Based on these initial observations, we surveyed 85 strains of *Coccidioide*s, representing isolates from the entire geographical range of *Coccidioides*, for growth rate differences between species at 37ºC and 28ºC. Initial investigations occurred at the University of Arizona, and subsequent studies occurred at Northern Arizona University (Table 1).

Table 1. Strain information

ID	Species	Geographical Origin[a]	Source	Testing Institution
CA22	*C. immitis*	California	University of Texas Health Science Center (UTHSC)	NAU

500	*C. posadasii*	Soil, Tucson, AZ	University of Arizona (UA)	UA
IL1	*C. posadasii*	Illinois	UTHSC	NAU
CA23	*C. immitis*	California	UTHSC	NAU
HS-I-000718	*C. posadasii*	Arizona	Flagstaff Medical Center (FMC)	NAU
GT164	*C. posadasii*	Texas	University of California Davis (UCD)	NAU
GT163	*C. immitis*	California	UCD	NAU
HS-I-000588	*C. posadasii*	Arizona	FMC	NAU
CA28	*C. immitis*	California	UTHSC	NAU
TX4	*C. posadasii*	Texas	UTHSC	NAU
HS-I-000235	*C. posadasii*	Arizona	FMC	NAU
TX1	*C. posadasii*	Texas	UTHSC	NAU
HS-I-000778	*C. posadasii*	Arizona	FMC	NAU
GT147	*C. immitis*	California	UCD	NAU
HS-I-000234	*C. posadasii*	Texas	FMC	NAU
CA30	*C. immitis*	California	UTHSC	NAU
HS-I-000547	*C. posadasii*	Arizona	FMC	NAU
HS-I-000233	*C. posadasii*	Arizona	FMC	NAU
GT166	*C. posadasii*	Texas	UCD	NAU
CA24	*C. immitis*	California	UTHSC	NAU
CA29	*C. immitis*	California	UTHSC	NAU
M211	*C. posadasii*	Central Mexico	Unidad de Micologia, UNAM	NAU
GT158	*C. posadasii*	Arizona	UCD	NAU
CA15	*C. immitis*	California	UTHSC	NAU
CA27	*C. immitis*	California	UTHSC	NAU
TX3	*C. posadasii*	Texas	UTHSC	NAU
CA20	*C. immitis*	California	UTHSC	NAU
RS	*C. immitis*	California	Common Laboratory Strain	NAU
Silveira	*C. posadasii*	California	Common Laboratory Strain	NAU
RMSCC2378	*C. posadasii*	Argentina	R. Negroni	UA
RMSCC2377	*C. posadasii*	Argentina	R. Negroni	UA
RMSCC2379	*C. posadasii*	Argentina	R. Negroni	UA
RMSCC3698	*C. immitis*	Barstow, California	Naval Hospital	UA
RMSCC3490	*C. posadasii*	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3505	*C. immitis*	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3506	*C. posadasii*	Coahuila, Mexico	I. Gutierrez	UA
RMSCC3472	*C. posadasii*	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3474	*C. immitis*	Michoacán, Mexico	I. Gutierrez	UA

RMSCC3475	*C. immitis*	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3476	*C. immitis*	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3478	*C. posadasii*	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3479	*C. immitis*	Michoacán, Mexico	I. Gutierrez	UA
RMSCC3377	*C. immitis*	Monterey, California	UCD	UA
RMSCC2343	*C. posadasii*	Nuevo Leon, Mexico	R. Diaz	UA
RMSCC2346	*C. posadasii*	Nuevo Leon, Mexico	R. Diaz	UA
RMSCC3738	*C. posadasii*	Piaui, Brazil	B. Wanke	UA
RMSCC3740	*C. posadasii*	Piaui, Brazil	B. Wanke	UA
RMSCC2127	*C. posadasii*	Texas	UTHSC	UA
RMSCC2133	*C. posadasii*	Texas	UTHSC	UA
RMSCC2234	*C. posadasii*	Texas	UTHSC	UA
RMSCC2102	*C. immitis*	San Diego, California	University of California San Diego (UCSD) Medical Center	UA
RMSCC2394	*C. immitis*	San Diego, California	UCSD Medical Center	UA
RMSCC2395	*C. immitis*	San Diego, California	UCSD Medical Center	UA
RMSCC3693	*C. immitis*	San Diego, California	Naval Hospital	UA
RMSCC3703	*C. immitis*	San Diego, California	UCSD Medical Center	UA
RMSCC3705	*C. immitis*	San Diego, California	UCSD Medical Center	UA
RMSCC3706	*C. immitis*	San Diego, California	UCSD Medical Center	UA
RMSCC2006	*C. immitis*	San Joaquin Valley	Kern County Public Health (KCPH)	UA
RMSCC2009	*C. immitis*	San Joaquin Valley	KCPH	UA
RMSCC2010	*C. immitis*	San Joaquin Valley	KCPH	UA and NAU
RMSCC2011	*C. immitis*	San Joaquin Valley	KCPH	UA
RMSCC2012	*C. immitis*	San Joaquin Valley	KCPH	UA
RMSCC2014	*C. immitis*	San Joaquin Valley	KCPH	UA

RMSCC2015	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2017	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2268	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2269	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2271	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2273	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2274	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2275	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2276	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2277	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2278	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2279	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2280	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC2281	*C. immitis*	San Joaquin Valley		KCPH	UA
RMSCC3480	*C. posadasii*	Sonora, Mexico		I. Gutierrez	UA
RMSCC3487	*C. posadasii*	Sonora, Mexico		I. Gutierrez	UA
RMSCC3488	*C. posadasii*	Sonora, Mexico		I. Gutierrez	UA
RMSCC1040	*C. posadasii*	Tucson, Arizona		UA	UA
RMSCC1043	*C. posadasii*	Tucson, Arizona		UA	UA
RMSCC1044	*C. posadasii*	Tucson, Arizona		UA	UA
RMSCC1045	*C. posadasii*	Tucson, Arizona		UA	UA
RMSCC3796	*C. posadasii*	Venezuela		G. San-Blas	

141 [a]*Often patient diagnosis location*

142 Observations were consistent between testing institutions, therefore data sets were
143 combined (S1 Fig). Using a mixed effect linear model, we showed a significant species-
144 specific difference for growth of the mycelial phase of the fungus based on temperature (Fig
145 2 and Table 2). Table 2 summarizes the estimated colony diameter for each predictor (species,
146 day, species per day), 95% confidence interval (CI), and p-value for each temperature specific
147 model. The radial growth rates of the two species differed significantly ($p<0.001$) at 37°C. At
148 this temperature, *C. posadasii* strains exhibited greater radial growth, with an increase in
149 diameter at a rate of 1mm/day, reaching double the diameter of *C. immitis* by day 16 (Fig 2
150 and Table 2). This was in contrast to growth at the lower temperature of 28°C, where *C.*
151 *immitis* grew more quickly than *C. posadasii*, although the difference was not statistically
152 significant (p-value = 0.072, Table 2). These findings were consistent for all days tested, and
153 represent differential phenotypes for both species. Thus, our analysis indicates that high
154 temperature is the important variable between species growth rate on solid media. This
155 phenotypic difference supports the molecular phylogenetic species designation and may
156 reflect adaptation of *C. immitis* to cooler environments, or possibly specific hosts.

157 **Fig 2. Radial growth rate of 85 isolates of *Coccidioides* demonstrates species-specific**
158 **response to temperature**. Each line represents the mean diameter (y-axis) for each isolate in
159 triplicate (46 *C. immitis* and 39 *C. posadasii)* at a given time point (x-axis). Dark lines
160 represent mean growth rate of each species. Radial growth was measured at day 5, 7, 9, 12,
161 14 and 16. There is a significant difference in growth rate (slope) in response to higher
162 temperature between species of *Coccidioides*. The radial growth rate of *C. immitis* is
163 decreased at a higher temperature 37ºC ($slope_{37}$ = 0.64 mm/day; 95% C.I. 0.51-0.78)
164 compared to *C. posadasii* ($slope_{37}$ = 1.82 mm/day; 95% C.I. 1.49-2.16). Both species appear

165 to tolerate 28ºC and grow at a similar rate (*C. immitis* $slope_{28}$ = 3.73 mm/day; 95% C.I. 3.53-
166 3.92, *C. posadasii*, $slope_{28}$ = 3.47 mm/day; 95% C.I. 2.98-3.90).

167 **Table 2. Temperature Specific Linear Models for Radial Growth Rate at 28ºC or 37ºC.**

	Colony Diameter at 28ºC			**Colony Diameter at 37ºC**		
Predictors	*Estimates*	*95% CI*	*p*	*Estimates*	*95% CI*	*p*
Species *C. immitis*	6.81	6.45 – 7.17	**<0.001**	6.11	5.91 – 6.30	**<0.001**
Species *C. posadasii*	6.56	6.17 – 6.95	**<0.001**	6.09	5.88 – 6.30	**<0.001**
Day	3.73	3.53 – 3.92	**<0.001**	0.64	0.51 – 0.78	**<0.001**
Species (*C. posadasii x* Day)	-0.26	0.55 – 0.02	0.072	1.18	0.98 – 1.38	**<0.001**
N[a]	85			85		

168 Summary of temperature specific linear models, for 28ºC and 37ºC, respectively. Colony
169 growth estimates for the predictors are species (y- intercept) and day (offset for y- intercept)
170 and species per day (slope). 95% confidence intervals (CI) for these estimates and p values
171 were used to compare each predictor. At 28ºC, *C. posadasii* grows 0.26 mm slower per day
172 than *C. immitis*. The difference in slope is not significant (p= 0.072). At 37ºC, *C. posadasii*
173 grows 1.18mm faster per day than *C. immitis*. The difference in slope (CI, 0.98-1.38 mm/day)
174 is statistically significant (p<0.001). [a]Number of individual strains.

Discussion

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

Functionally, this phenotype is similar to a classic temperature sensitive (ts) conditional mutant, such that *C. immitis* exhibits normal growth at permissive temperature, and significantly slower growth under stressful conditions. It is possible that *C. immitis* could be restored to normal growth at 37°C by gene replacement with appropriate *C. posadasii* alleles if candidate genes were identified. Several genes and pathways have been described in *Aspergillus fumigatus* related to thermotolerance [51]. For example, the observed phenotype could be due to mutations in a heat shock protein (Hsp). Hsps are activated in response to changes in temperature and regulate cellular processes associated with morphogenesis,

197 antifungal resistance, and virulence by triggering a wide array of cellular signaling pathways
198 [50, 59]. Hsps are activated by a heat shock transcription factor (Hsf) that acts as a
199 thermosensor, regulating the Hsps at specific growth temperatures [60]. Several studies have
200 shown that *Coccidioides* up-regulates heat shock proteins Hsp20 and Hsp9/12 at high
201 temperature during the parasitic lifecycle while down-regulating Hsp30 and Hsp90 [61-64].
202 Further investigation of Hsps and Hsfs in *Coccidioides* could elucidate mechanisms of the
203 species-specific thermotolerant behavior observed in this study. Alternatively, many classical
204 ts mutants occur in genes required for normal cellular growth and are due to single amino acid
205 changes that affect protein function or stability at the restrictive temperature. For example, a
206 number of colonial temperature sensitive (*cot*) mutants have been identified in *Neurospora*
207 *crassa*. The *N. crassa cot-1* mutant has been studied in greatest detail, and the ts defect is due
208 to a SNP causing a single amino acid change in a Ser/Thr protein kinase required for normal
209 hyphal extension, thus resulting in restricted growth at normally permissive temperatures
210 above 32°C [65, 66]. Finally, recent work in *Saccharomyces* indicates that mitochondrial
211 genotypes are associated with heat tolerance [67]. The mitochondrial genomes of the two
212 species of *Coccidioides* are also distinct, and thus mitochondrial function is another potential
213 mechanism controlling thermotolerance in *Coccidioides*.

214 The source of the genotypic variation driving the observed phenotype may be
215 attributable to a stochastic event, such as a founder effect or population bottleneck 10-12
216 MYA, which is the estimated time the two species have been separated [5, 68]. Alternatively,
217 the observed pattern may be due to selection pressure from a specific environment, host, or
218 directly associated with virulence. Thus, the observed differential thermotolerance may relate
219 to the saprobic phase of the lifecycle and reflect adaptation to specific environments. A pattern

of alternating wet-dry conditions has been related to Valley fever incidence across the southwestern U.S. [69-74]. It has been proposed that fungal growth occurs during brief periods of heavy moisture during monsoon and winter rainy seasons in the Southwest, which are followed by prolific conidia production when warm temperatures and low rainfall desiccate soils and increase dispersal via dust (the "grow and blow" hypothesis) [26, 70, 75]. Additionally, during high temperature periods, it is hypothesized that the surface soil is partially sterilized and many competitors are removed, but *Coccidioides* spores remain viable [25]. Another hypothesis is that *C. posadasii* may be better adapted to growth in the high soil temperatures observed in the southwestern deserts compared to the California endemic *C. immitis*. Maricopa, Pinal and Pima counties harbor the highest coccidioidomycosis case rates in Arizona due to *C. posadasii*, and according to the National Centers for Environmental Information [76], the annual mean temperature (1901-2000) were 20.7°C, 19.8°C and 19.2°C, respectively. On the other hand, Fresno, King and Kern counties, which harbor the highest coccidioidomycosis case rates in California due to *C. immitis*, had annual mean temperatures of 12.4°C, 16.9°C and 15.8°C, respectively. The difference in 100-year average annual mean temperature between highly endemic areas of Arizona and California supports our hypothesis that *C. posadasii* is more adapted to higher temperatures compared to *C. immitis*. Alternatively, a preferred host species may vary in normal body temperature, in accordance with the endozoan small mammal reservoir hypothesis proposed by Barker and Taylor [77]. Interestingly, a decline in mean human body temperature (~1.6%) has recently been reported [78]. Whether this impacts coccidioidomycosis rates is unknown.

Published literature to date suggests that disease outcomes are related primarily to host-specific factors [36, 37, 79], and certainly, host genetic background can impact disease

243 progression. We propose that pathogen-specific variation may also contribute to capricious
244 disease outcomes in coccidioidomycosis patients. Currently, species-specific virulence is not
245 well-documented in *Coccidioides* research, but has been suggested [1, 12]. This is in part due
246 to the use of a few characterized laboratory strains of *Coccidioides* for most hypothesis testing,
247 primarily strains Silveira, C735 and RS [61-64, 81-83]. Therefore, connecting phenotypic
248 dissimilarity to established genetic variation using genome-wide association studies could
249 provide insight into unique characteristics of these genetically distinct pathogens.

250 In summary, we have identified a significant phenotypic difference between *C. immitis*
251 and *C. posadasii.* Although growth rate on YE media at two temperatures is the only
252 characteristic we explicitly tested, there are certain to be more phenotypic differences between
253 species, and possibly between populations. This, coupled with the recent availability of the
254 genome sequence of multiple strains for both fungal species, may allow comparative genomic
255 approaches to elucidate candidate genes for thermotolerance regulation in *Coccidioides* and
256 closely related Onygenales [6].

257 ## Methods

258 **Strains and Media.** 39 *C. posadasii* strains and 46 *C. immitis* strains used in this study are
259 primarily human patient isolates archived by various institutions, as detailed in Table 1 [5, 7,
260 27, 84]. These strains represent both the full geographic range of the two species, and the
261 proposed geographically distinct sub-populations [5, 7]. Strains were grown on 2xGYE media
262 (2% glucose, 1% yeast extract, 1.5% agar w/v) to supply initial plugs to inoculate plates for
263 growth analysis. Yeast Extract (YE) media (0.5% yeast extract, 1.5% agar w/v) was used for

264 growth experiments. Flagstaff Medical Center isolates were collected under IRB No. 764034
265 through Northern Arizona Healthcare as part of the Northern Arizona University Biobank.
266 **Growth Conditions and Measurements.** Colonies were started by spreading approximately
267 10^6 arthroconidia over the entire surface of a 2xGYE plate to create a lawn of mycelium to be
268 transferred to initiate the thermotolerance experiment; this allowed measurement of colonial
269 growth and not spore germination differences. After five days of growth at 25ºC, 7mm
270 diameter mycelial plugs were subcultured to the center of YE plates using a transfer tool
271 (Transfertube® Disposable Harvesters, Spectrum® Laboratories). Three replicates of each
272 strain were plated for each experiment. All plates (100mm x 15mm BD Falcon 1015) were
273 sealed with gas permeable seals (labtape form TimeMed Labeling Systems, Inc or Key
274 Scientific plate seals) for safety. Plates were placed in temperature-controlled incubators at
275 either 28ºC or 37ºC in the dark under ambient humidity (30-50% RH) and CO_2 (0.1%)
276 conditions. Plate stacks were rotated from top to bottom and repositioned in the incubator with
277 each measurement timepoint to reduce effects of environmental variation within the
278 incubators. For measurement of radial growth, the diameter of each colony was measured in
279 mm at 5, 7, 9, 12, 14, and 16 days post-subculture. The initial experiment proceeded at
280 University of Arizona (UA) and subsequent testing with a new set of isolates occurred at
281 Northern Arizona University (NAU). Details for strains tested at each institution are listed in
282 Table 1 and all raw measurement data are available in S1 File.
283 **Statistical Analysis.** To estimate the mean growth rate for each species over the two-week
284 period a mixed effect linear model for each temperature was constructed using the lme4
285 package in R version 3.6.2 [85, 86]. Initially, data sets were divided by institution and after
286 concluding that parameters of interest were not impacted by collection site the data sets were

287 combined. In the temperature specific models, the factors "day" and "species" were assumed
288 to be fixed linear effects, and individual isolate response for each day was considered to be a
289 normally distributed random effect as appropriate in a longitudinal study. Thus, the response
290 variable of colony diameter was modeled with fixed effects and a random effect to determine
291 if growth rates varied between strains at either 28ºC and 37ºC. Shapiro-Wilk test (p-value <
292 0.001) shows that residuals are not normally distributed. However, the large sample size and
293 overall residual structure support that a linear model is the most appropriate for this data set.
294 In addition, bootstrapping using the boot package in R [87, 88] was used to estimate 95%
295 confidence intervals (CIs) for growth rates and other fixed effects (nsim=2,000). All bootstrap
296 parameters were similar and support model estimates. A comparison between bootstrapped
297 CIs and CIs constructed using the linear model can be found in S1 Table and S2 Table.

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302

303 ## Supporting information

304 **S1 Fig. Growth of *C. immitis* and *C. posadasii* on YE media at NAU and UA.** Seven mm
305 diameter plugs were sub-cultured onto yeast extract plates and radial growth was documented
306 over sixteen days. (A) Radial growth measurements at 28ºC and 37ºC for 85 isolates in

triplicate, at both institutions. (B) Representative samples of phenotypic variation observed between species on day sixteen for both NAU and UA experiments.

S1 Table. Comparison of 28ºC Linear Model and Bootstrap Values. Comparison of 28ºC linear model and bootstrap 95% confidence intervals. Bootstrapping conducted using the boot package in R.

S2 Table. Comparison of 37ºC Linear Model and Bootstrap Values. Comparison of 37ºC linear model and bootstrap 95% confidence intervals. Bootstrapping conducted using the boot package in R.

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

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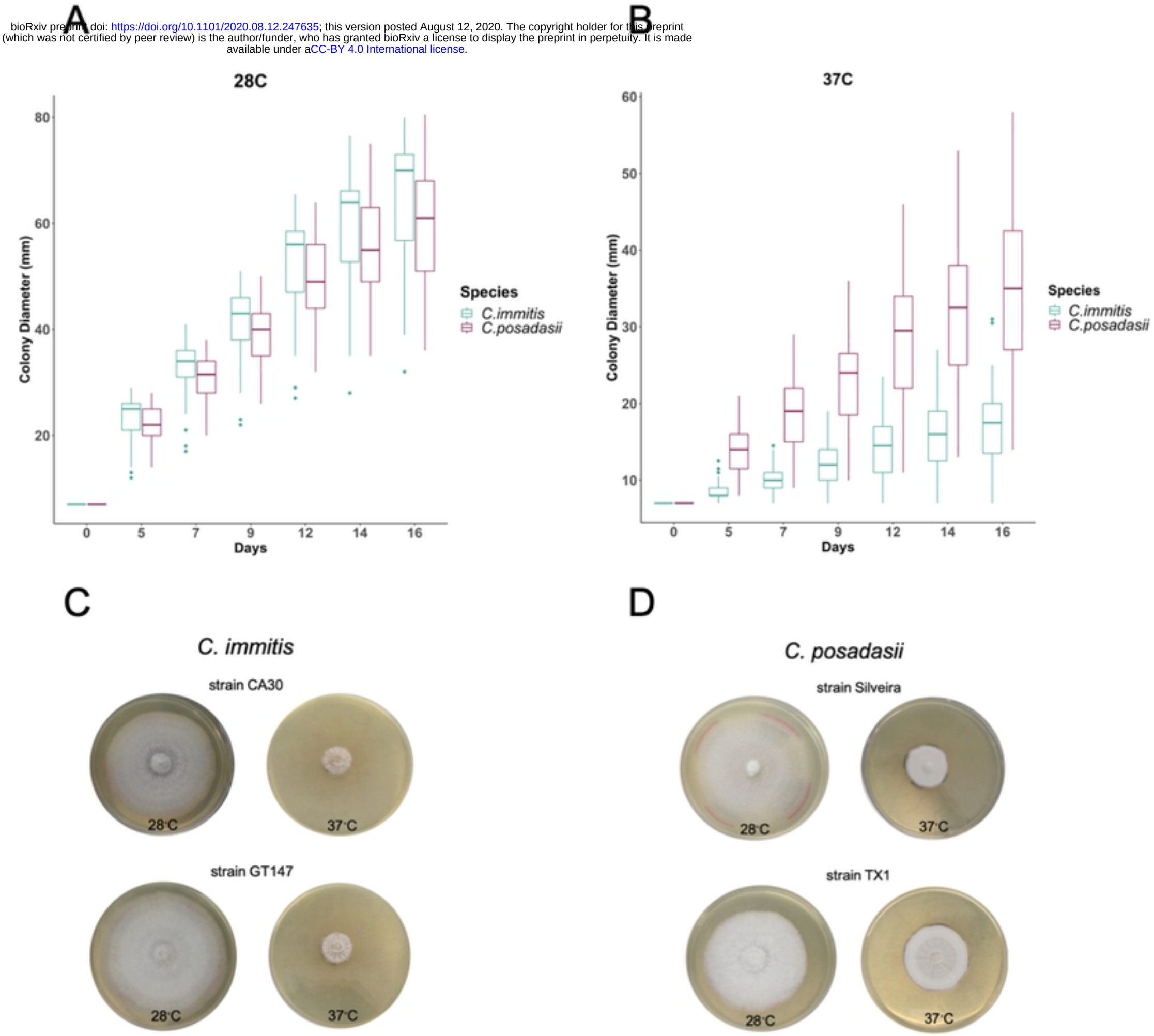

Figure 1

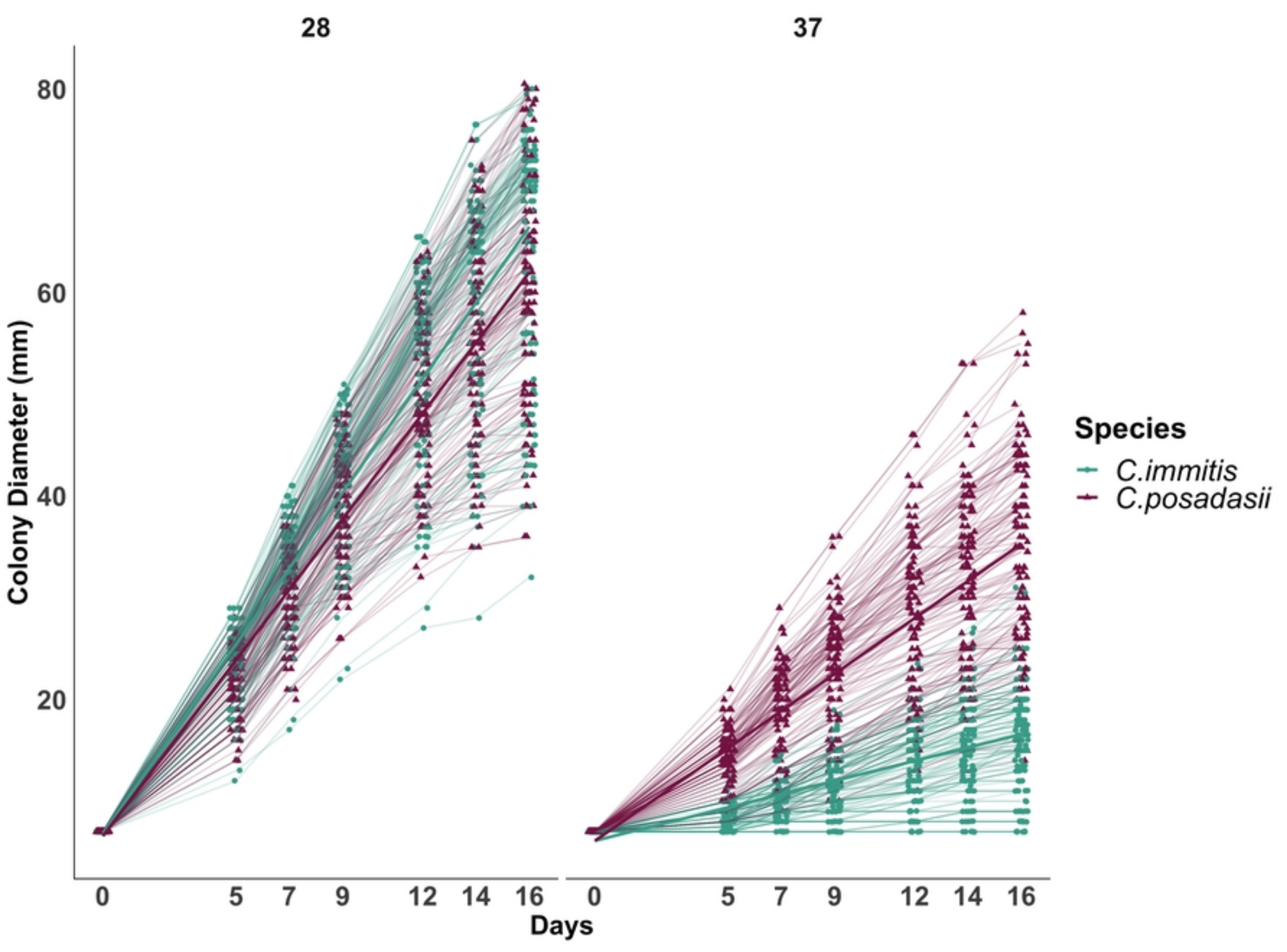
28
37
80
60
40
20
Colony Diameter (mm)
0
5
7
9
12
14
16
Days
Species
C.immitis
C.posadasii

Figure 2