Lifecycle dominates the volatilome character of the dimorphic fungus *Coccidioides* spp.

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Running Title: Lifecycle dominates the volatilome of *Coccidioides*

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16 **ABSTRACT**

17 Valley fever (coccidioidomycosis) is an endemic fungal pneumonia of the North and South
18 American deserts. The causative agents of Valley fever are the dimorphic fungi *Coccidioides*
19 *immitis* and *C. posadasii*, which grow as mycelia in the environment and spherules within the
20 lungs of vulnerable hosts. The current diagnostics for Valley fever are severely lacking due to
21 poor sensitivity and invasiveness, contributing to a 23-day median time-to-diagnosis, and
22 therefore new diagnostic tools are needed. We are working toward the development of a breath-
23 based diagnostic for coccidioidomycosis, and in this initial study we characterized the volatile
24 metabolomes (or volatilomes) of *in vitro* cultures of *Coccidioides*. Using solid-phase
25 microextraction and comprehensive two-dimensional gas chromatography coupled to time-of-
26 flight mass spectrometry (GC×GC–TOFMS), we characterized the VOCs produced by six strains
27 of each species during mycelial or spherule growth. We detected a total of 353 VOCs that were
28 at least two-fold more abundant in a *Coccidioides* culture versus medium controls and found the
29 volatile metabolome of *Coccidioides* is more dependent on growth phase (spherule versus
30 mycelia) than on the species. The volatile profiles of *C. immitis* and *C. posadasii* have strong
31 similarities, indicating that a single suite of Valley fever breath biomarkers can be developed to
32 detect both species.

33

34 **IMPORTANCE**

35 Coccidioidomycosis, or Valley fever, causes up to 30% of community-acquired pneumonias in
36 endemic and highly populated areas of the US desert southwest. The infection is difficult to
37 diagnose by standard serological and histopathological methods, which delays an appropriate
38 treatment. Therefore, we are working toward the development of breath-based diagnostics for

Valley fever. In this study, we characterized the volatile metabolomes of six strains each of *Coccidioides immitis* and *C. posadasii*, the dimorphic fungal species that cause Valley fever. By analyzing the volatilomes during the two modes of growth for the fungus—mycelia and spherules—we observed that the lifecycle plays a significant role in the volatiles produced by *Coccidioides*. In contrast, we observed no significant differences in the *C. immitis* versus *C. posadasii* volatilomes. These data suggest that lifecycle, rather than species, should guide the selection of putative biomarkers for a Valley fever breath test.

INTRODUCTION

Coccidioidomycosis, or Valley fever, is a disease that is endemic to the deserts of the western United States, Mexico, and Central and South America and is responsible for an estimated 350,000 new infections per year (1). In endemic and highly populated areas of the US, e.g., Phoenix and Tucson, Arizona and the San Joaquin Valley in California, up to 30% of community-acquired pneumonias may be caused by Valley fever, and with growing populations in these regions, Valley fever cases in the US are expected to climb (2). The causative agent of Valley fever is the opportunistic pathogen *Coccidioides*, a genus of dimorphic fungus comprised of two species, *Coccidioides immitis* and *C. posadasii*. In the environment, *Coccidioides* spp. grow in the soil during rainy periods as mycelia, which become dormant and form arthroconidia during dry periods. As the soil is disturbed, the arthroconidia can become airborne, and when inhaled, the fungus can initiate a parasitic lifecycle as spherules within the lungs of a susceptible host, causing pneumonia. The median duration of human Valley fever pneumonia is 120 days, costing $93,734 per person in direct and indirect costs (2, 3). Approximately three quarters of the

61 diagnosed cases of Valley fever require antifungal therapy (4), and 1% of those infected will
62 experience disseminated disease, with a third of those being fatal (4).
63
64 A definitive diagnosis for Valley fever requires a positive fungal culture or histological
65 examination of tissue or bodily fluid. However, due to the invasiveness of obtaining suitable lung
66 specimens for culture or histology, serological testing for antibodies against *Coccidioides* is the
67 most commonly performed diagnostic test. Multiple serological tests are available for IgM, IgG,
68 or complement-fixing antibodies, as well as an enzyme-linked immunoassay that uses
69 proprietary coccidioidal antigens. However, these tests lack sensitivity for coccidioidomycosis
70 (5, 6). Due to the difficulty in diagnosing Valley fever, it is often mistaken for bacterial
71 pneumonia and inappropriately treated with antibiotics (7-10). The lack of a suitable diagnostic
72 strongly contributes to an unacceptably long 23-day median time-to-diagnosis (2).
73
74 Breath-based diagnostics are a promising novel approach for diagnosing respiratory infections.
75 Numerous *in vitro* studies have demonstrated that bacterial and fungal respiratory pathogens
76 have unique volatile metabolome (or *volatilome*) signatures that can be used to differentiate and
77 identify them to the genus, species, and strain level (11). Thus far, relatively few studies have
78 specifically focused on identifying breath biomarkers for fungal lung diseases. *In vitro* analyses
79 of the volatiles produced by *Aspergillus* spp. have shown that fungi produce many compounds
80 that are also frequently detected in bacterial cultures, e.g., (alcohols) ethanol, propanol, (ketones)
81 acetone, 2-nonanone, 2-undecanone, 2,3-butanedione, (sulfides) dimethyl disulfide, dimethyl
82 trisulfide, and (pyrazines) 2-methyl pyrazine, and 2,5-dimethyl pyrazine (12-16). However,
83 *Aspergillus* also produces a wider variety of monoterpenes, sesquiterpenes, and terpenoids,

which are less common in bacterial volatilomes (14, 17). With this knowledge, Koo et al. used a biologically guided approach to identify volatile biomarkers of pulmonary invasive aspergillosis (IA), focusing on terpenes and terpenoids produced *in vitro* as putative biomarkers for a breath test (17). They determined that the *A. fumigatus in vitro* volatilome contained a distinctive combination of monoterpenes and sesquiterpenes, and in breath the sesquiterpenes were the most specific for differentiating patients with IA versus other pulmonary infections (17). This example for *A. fumigatus* establishes a proof-of-concept that direct detection of fungal metabolites in breath can be used as a noninvasive, species-specific approach to identify the underlying microbial cause of pneumonia.

We are taking an *in vitro*-guided approach toward developing a Valley fever breath test by first building a catalog of volatile compounds produced by *C. immitis* and *C. posadasii*. In this study, we have cultured six strains of each species and used solid-phase microextraction and comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC–TOFMS) to characterize the volatile organic compounds (VOCs) produced during mycelial or spherule growth. We expected to find significant differences in the VOCs between species and between lifecycles, but also posited that there would be a subset of VOCs that are common to *Coccidioides* spp. during spherule growth, which will serve as our candidate biomarkers for breath test development.

RESULTS

Coccidioides isolates in this study

106 The *Coccidioides* genus is widely distributed throughout North and South America. The genus is
107 composed of two geographically (18) and genetically distinct species (18, 19), *C. posadasii* and
108 *C. immitis*, which are further divided into five well-defined populations (20-22). *C. posadasii* has
109 a more extensive biogeographic distribution and is divided into three populations, occurring in
110 Texas/Venezuela, Mexico/South America, and Arizona. The species *C. immitis* is divided into
111 two populations found in central and southern California, and recently Washington State,
112 representing a newly discovered third distinct population (23, 24). Representative members of all
113 populations cause disease in humans and other mammals (20-22). Due to the magnitude of
114 observed genetic variation and geographic distribution between species, we hypothesized that
115 metabolomic variation exists. To capture diversity across the volatilome between and within
116 species, we included a total of twelve isolates from both species, representing four populations,
117 all of which were isolated from humans (Table 1).
118
119 **Table 1.** *Coccidioides* spp. strains used in this study. All isolates are from human infections.

Species	Strain	Population*	ATCC	NCBI**
C. posadasii	Silveira	AZ	NR-48944	ABAI00000000.2
	B3221	AZ		NA
	B3222	AZ		NA
	RMSCC2343	TX/MEX/SA		SRR3468064
	RMSCC3506	TX/MEX/SA		SRR3468053
	GT-166	TX/MEX/SA		NA
C. immitis	RS	SDMX	NR-48942	AAEC00000000.3
	RMSCC2395	SDMX	NR-48938	NA
	RMSCC3505	SDMX		NA
	RMSCC2006	SJV	NR-48934	NA
	RMSCC2009	SJV		SRR3468015
	RMSCC2010	SJV	NR-48935	NA

120 *Populations are defined as *C. posadasii* collected from Arizona (AZ), *C. posadasii* collected
121 from Texas/Mexico/South America (TX/MEX/SA), *C. immitis* collected from San Diego,
122 California, and Mexico (SDMX), and *C. immitis* collected from San Joaquin Valley, California
123 (SJV), as previously published (25). **Published genotype data, if available (21).

124 **Characteristics of the *Coccidioides* volatilome by species and lifecycle**

125 We cultured each *Coccidioides* isolate in biological triplicate using temperatures and oxygen
126 concentrations that induce mycelial or spherule growth, but using the same culture medium,
127 facilitating direct comparisons between the VOCs produced during the two lifecycles. Using
128 solid-phase microextraction to adsorb VOCs from the headspace of fungal culture filtrates, and
129 GC×GC–TOFMS to analyze the volatilomes, we detected a total of 353 chromatographic peaks
130 that were at least two-fold more abundant in a *Coccidioides* culture (n = 72) versus the medium
131 controls (n = 6) (Table S1). Of the 353 volatiles detected in *Coccidioides* cultures, 28 were
132 assigned putative names based on mass spectral and chromatographic data, which included 13
133 compounds previously associated with human and environmental fungal pathogens (26) (Table
134 2). For the unnamed compounds, we assigned chemical classifications to 45 of them based on a
135 combination of mass spectral and chromatographic characteristics (Table S1).

136

137 The total number of volatiles produced by *C. posadasii* versus *C. immitis* were similar, at 291
138 versus 309, respectively (Figure 1; Table S2). Within each species, the two lifecycles are quite
139 divergent in their volatilomes, sharing less than a third of the total volatilome (Figure 1, A and
140 B). However, within each lifecycle, the two species share many similarities, with more than half
141 of the VOCs produced by both *C. posadasii* and *C. immitis* (Figure 1, C and D). In aggregate
142 across the genus, or subdivided by species, the spherule volatilome is larger than the mycelial
143 volatilome, but this appears to be driven by considerable differences in volatilome sizes observed
144 in a few isolates (*C. posadasii* B3221, GT-166; *C. immitis* RS; Table S2).

145

146 **Table 2.** Named *Coccidioides* spp. volatiles and their previous reports in other fungal taxa.

VOC	Compound	Functional class	CAS	Fungal taxa*							References
				1	2	3	4	5	6	7	
29	acetonitrile	nitrogen-containing	75-05-8								
97	acetic acid	carboxylic acid/ester	64-19-7	x	x						(27, 28)
147	acetoin	ketone	513-86-0		x						(28)
159	2-methyl-1-butanol	alcohol	137-32-6	x	x		x	x	x	x	(29-33)
195	hexanal	aldehyde	66-25-1	x	x						(28, 29, 34)
196	4-methyl-3-penten-2-one	ketone	141-79-7								
206	N,N-dimethyl-formamide	nitrogen-containing	68-12-2								
239	1-hexanol	alcohol	111-27-3		x						(29)
255	2-hexen-1-ol	alcohol	2305-21-7								
270	cyclohexanone	ketone	108-94-1	x	x	x			x		(31, 35)
299	1-ethyl-4-methyl-benzene	aromatic hydrocarbon	622-96-8								
320	2,5-hexanedione	ketone	110-13-4								
332	2-pentylfuran	heteroaromatic	3777-69-3	x			x			x	(32, 33, 36-39)
354	5-methyl-3-heptanone	ketone	541-85-5								
359	β-pinene	monoterpene	127-91-3	x					x		(14, 17, 31, 34)
364	4,6-dimethyl-2-heptanone	ketone	19549-80-5								
383	limonene	monoterpene	138-86-3	x	x		x		x	x	(14, 17, 29-31, 33, 34, 40-42)
386	1-methyl-3-(1-methylethyl)-benzene	aromatic hydrocarbon	535-77-3								
411	2-chloro-phenol	aromatic alcohol	95-57-8								

428	2,4,6-trimethlpryidine	nitrogen-containing, aromatic	108-75-8								
435	3-undecene	hydrocarbon	1002-68-2								
456	benzeneacetaldehyde	aromatic aldehyde	122-78-1		x				x		(29, 40)
502	1,3-diethenyl-benzene	aromatic	108-57-6								
545	2-decanone	ketone	693-54-9							x	(33)
554	2-(2-butoxyethoxy)-ethanol	alcohol	112-34-5								
555	decanal	aldehyde	112-31-2	x	x						(29, 34)
820	2-butyl-1-octanol	alcohol	3913-02-8								
955	hexadecane	hydrocarbon	544-76-3	x							(13)

*Fungal taxa: 1 = *Aspergillus* spp.; 2 = *Candida* spp.; 3 = *Cladosporium cladosporoides*; 4 = *Fusarium* spp.; 5 = *Mucor* spp.; 6 = *Penicillium* spp.; 7 = Other spp., including environmental fungal species, *Tricoderma* spp., *Stropharia rugosoannulata*, *Verticillium longisporum*

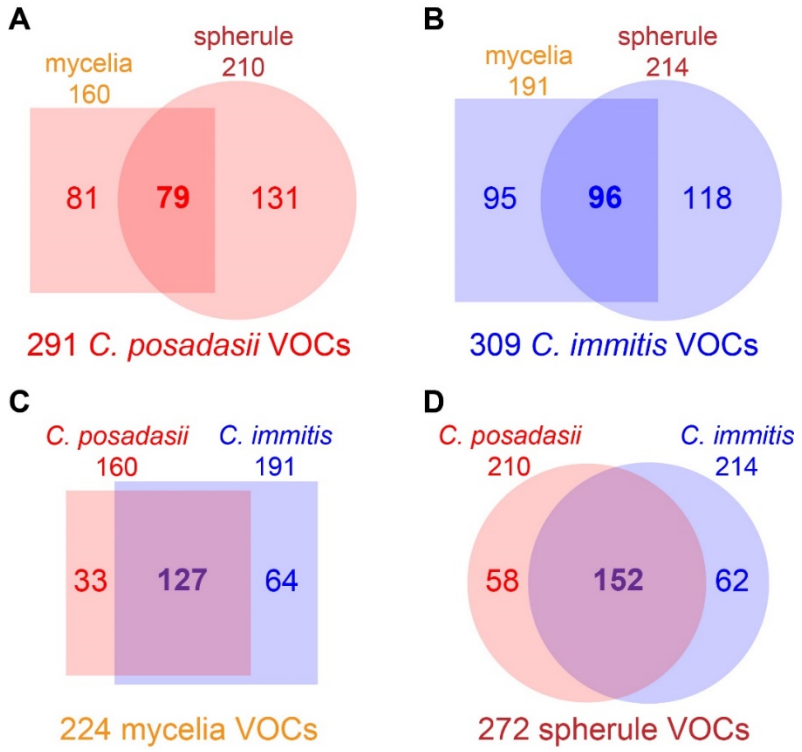

Figure 1. The numbers of analytes in at least two-fold greater abundance in a *Coccidioides* culture versus the media controls that are shared and unique among **A**) *C. posadasii* mycelia

154 (square) vs. spherules (circle); **B**) *C. immitis* mycelia (square) vs. spherules (circle); **C**) mycelia
155 of *C. posadasii* (red) vs. *C. immitis* (blue); and **D**) spherules of *C. posadasii* (red) vs. *C. immitis*
156 (blue).

157

158 Analyzing the relative abundances of individual analytes, we observed some statistically-
159 significant differences in VOCs produced during the two lifecycles, but not between the two
160 species. We performed a Mann-Whitney *U*-test using Benjamini-Hochberg false discovery rate
161 (FDR) correction comparing all spherule and mycelial VOCs, and identified 43 that were
162 significantly different in abundance ($p < 0.05$) (Figure 2). Of the 43 volatiles, 32 of them were
163 higher in abundance in spherule compared to 11 in mycelia, which mirrors the overall difference
164 in the size of the volatilomes (Figure 1). Among the VOCs detected in higher abundance in one
165 lifecycle versus another were 1-methyl-3-(1-methylethyl)-benzene and hexanal, which are
166 associated with mycelia cultures, while 2-(2-butoxyethoxy)-ethanol, cyclohexanone, 2-hexen-1-
167 ol, and 2,4,6-trimethlpryidine were associated with spherule cultures. There were no VOCs that
168 were significantly different in relative abundance between *C. posadasii* and *C. immitis*.

169

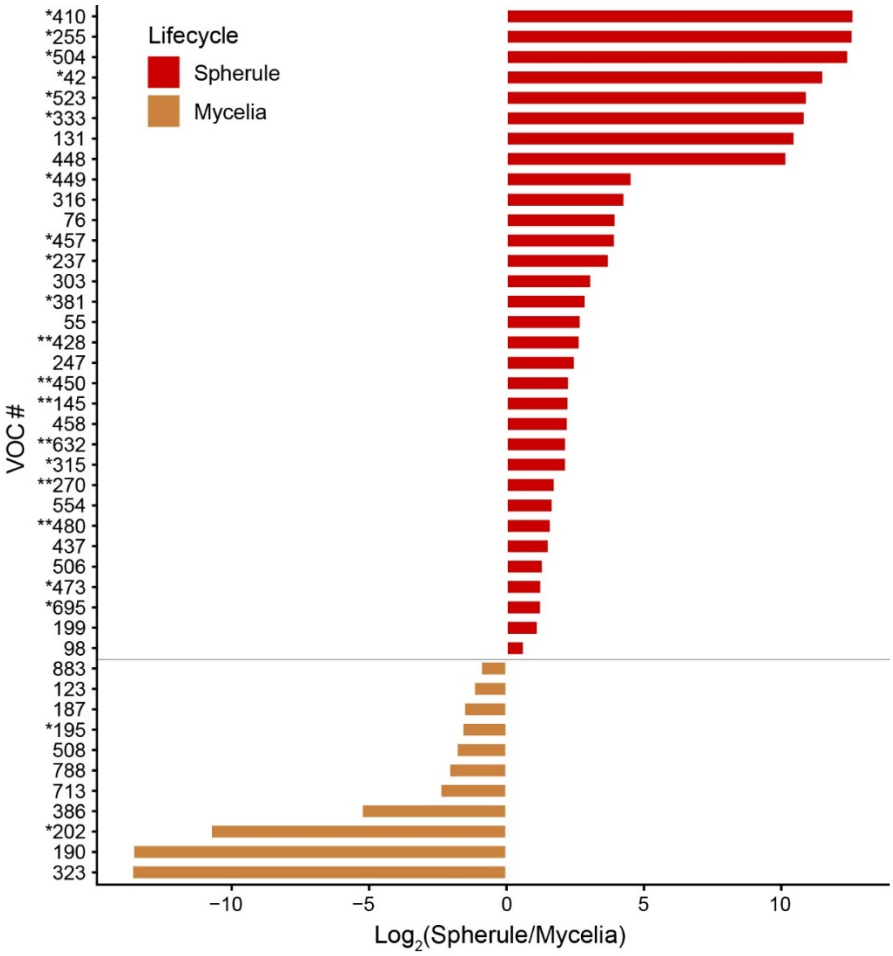

Figure 2. Forty-three volatiles significantly different in abundance between lifecycles ($p < 0.05$), expressed as $\log_2$ fold change in peak abundance. Maroon and tan represent spherule and mycelia peaks, respectively. Volatiles marked with an asterisks indicate greater statistically-significant differences (* $p < 0.001$, ** $p < 0.0001$). Compound IDs: 195 = hexanal; 255 = 2-hexen-1-ol; 270 = cyclohexanone; 386 = 1-methyl-3-(1-methylethyl)-benzene; 428 = 2,4,6-trimethlpryidine; 554 = 2-(2-butoxyethoxy)-ethanol.

Most (> 70%) of the spherule and mycelial VOCs detected in this study were produced by four or fewer *Coccidioides* strains in each lifecycle. Of the 272 spherule volatiles that were detected, only 35 (13%) were detected in at least two-thirds of all spherule cultures (Figure 3A), 12 of which were also significantly more abundant in spherule versus mycelia cultures. Nineteen of 224 mycelia volatiles (8%) were detected in at least two-thirds of all mycelia samples (Figure 3B), and only two were significantly different between lifecycles (VOC 199 and 428). However,

185 both volatiles were produced in higher relative abundance in spherules. Only two VOCs, 39 and
186 748, were detected in two-thirds of both spherule and mycelia samples. Interestingly, the relative
187 abundance of a VOC was usually consistent across all strains. For example, in every spherule
188 culture in which limonene (VOC 383) was detected, it was at a relative concentration between
189 two and tenfold greater than media (Figure 3A).

190

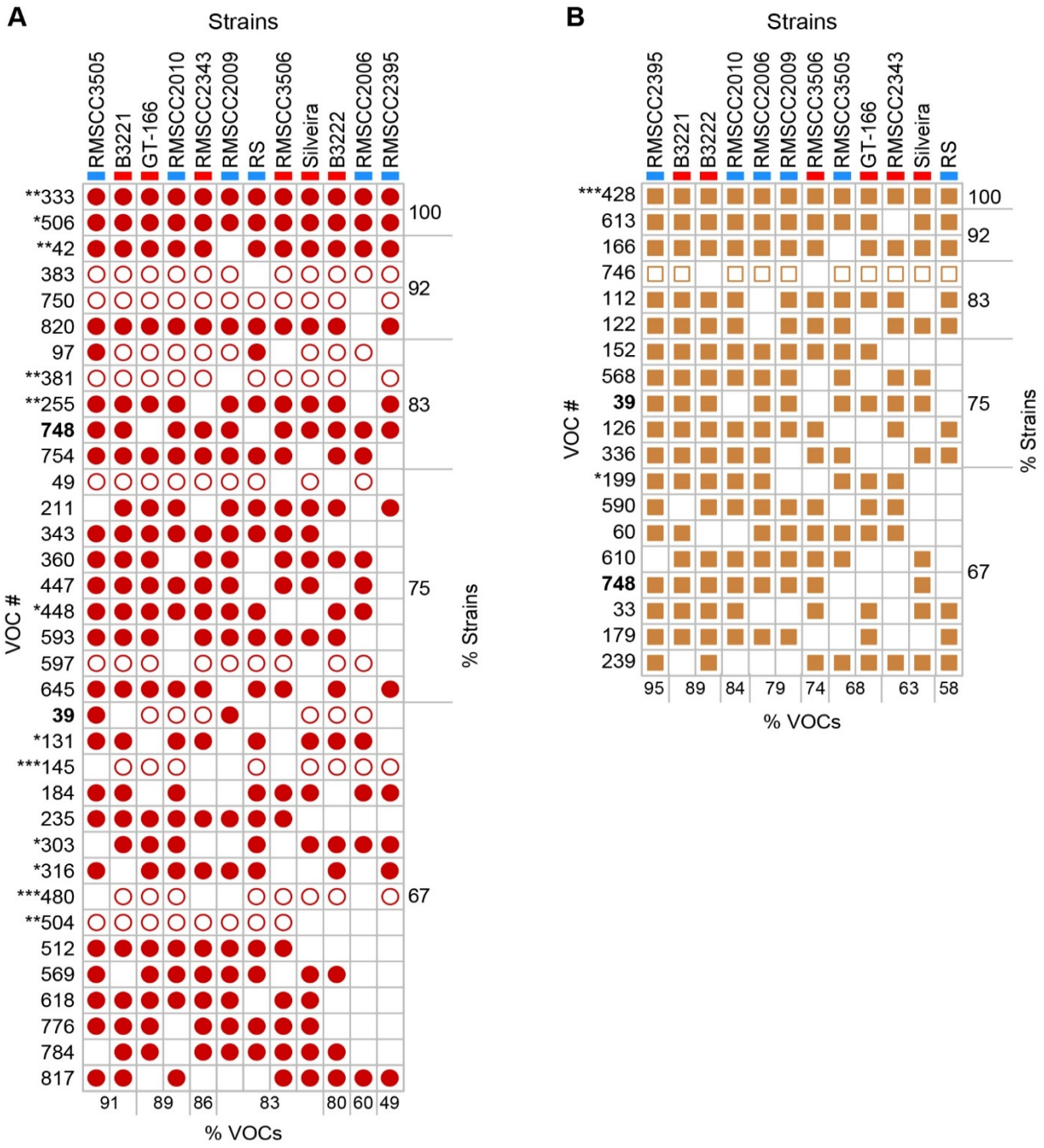

Figure 3. Volatiles detected in at least two-thirds of all *Coccidioides* strains in spherule (A) or mycelial (B) cultures, with VOCs in bold detected in both lifecycles. Open shapes indicate VOCs that are at least 2-fold greater in relative abundance compared to media blanks, and solid shapes indicate VOCs that are at least 10-fold greater in relative abundance. Volatiles marked with an asterisks indicate statistically-significant differences in abundance between lifecycles (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$). Strains are color-coded as *C. immitis* (blue) and *C. posadasii* (red). The percentages of listed VOCs detected in each strain, and the percentages of strains producing each VOC are noted at the bottom and right side of each chart, respectively. Compound IDs: 97 = acetic acid; 239 = 1-hexanol; 255 = 2-hexen-1-ol; 383 = limonene; 428 = 2,4,6,-trimethyl-pyridine; 820 = 2-butyl-1-octanol.

202

203 **Multivariate analyses of the *Coccidioides* volatilome**

204 Principal components analysis (PCA), using the 78 cultures and media blanks as observations
205 and 353 *Coccidioides* VOCs as variables, shows that the volatilomes of individual strains and
206 culture conditions are highly reproducible (Figure S1A), and that they cluster by lifecycle, not by
207 species (Figure 4). This observation is reinforced when we include only mycelia or spherules in
208 the PCA, where no separation between species is observed (Figure S1, B and C), or only include
209 *C. immitis* or *C. posadasii*, where we still observe clear separation by lifecycle (Figure S1, D and
210 E). Evaluating these subsets of the data, some interesting variations between strains emerge. The
211 type strain for *C. immitis* is RS; however, in both mycelial and spherule lifecycles, we observe
212 that its volatile metabolome is quite unique from the other *C. immitis* strains (Figure S1). *C.*
213 *posadasii* also has a metabolic outlier – RMSCC3506 – but only during mycelial growth (Figure
214 S1, B and E). These outliers cannot be fully explained by the size of the volatilomes as being
215 abnormally large or small (Table S2).

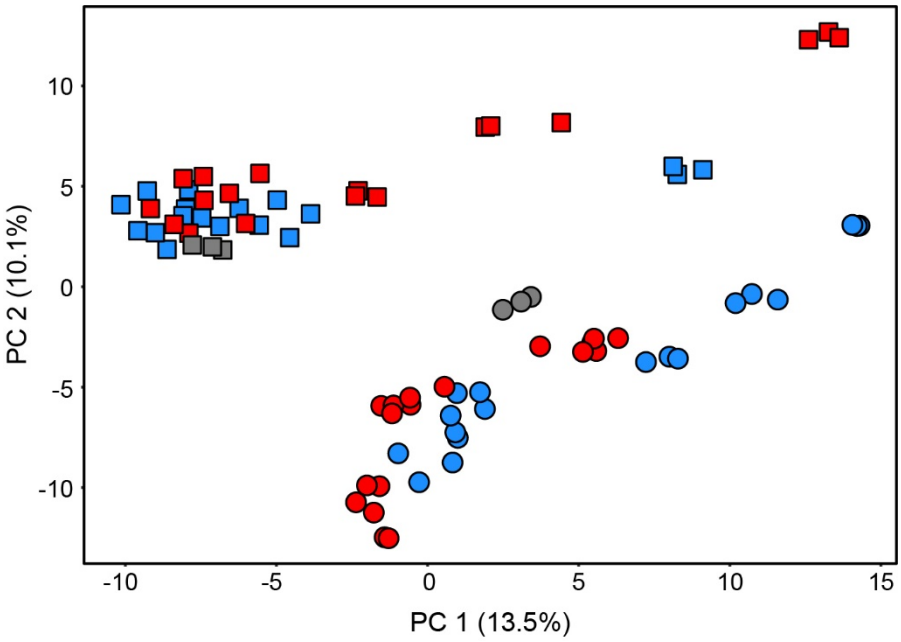

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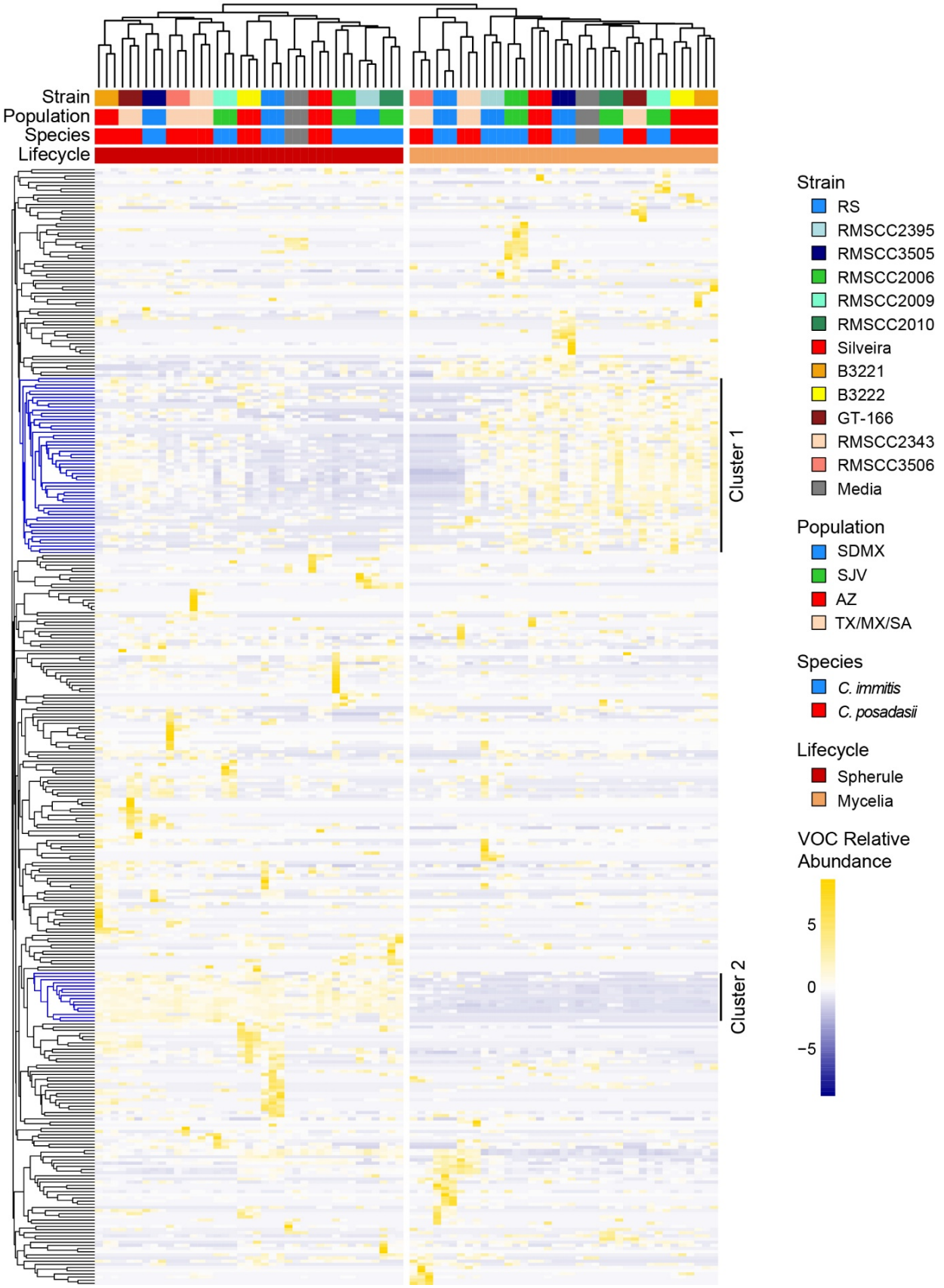
Figure 4. Principal components analysis (PCA) score plot using 353 VOCs as features, produced by *C. immitis* (blue) and *C. posadasii* (red) when cultured in conditions that induced spherule (circle) or mycelial (square) morphologies. Blank media are shown in gray. Fungal cultures and media blanks were analyzed in triplicate, yielding 72 fungal and 6 blank observations. PCA score plots with the observations colored by strain are in Figure S1.

Though lifecycle and strain differences in the *Coccidioides* volatilome are observable in the PCA, less than a quarter of the total variance is captured by the first two PCs. Thus, we also used hierarchical clustering analysis (HCA) to compare the *Coccidioides* volatilomes (Figure 5). The HCA reinforces our finding that lifecycle is the most significant contributor to the *Coccidioides* volatilome, and there are not global similarities in the volatilomes within each species, or within populations of the species. There are two main clusters of VOCs driving the separation of the mycelia and spherule lifecycles in the HCA. Cluster 1 includes 56 VOCs, which are generally present in a higher abundance in mycelia samples compared to spherule samples, five of which are significantly different between lifecycles ($p < 0.05$ with Benjamini-Hochberg FDR correction; Table S1). In Cluster 1, seven compounds are identified: 3-undecene, 1-ethyl-4-methyl-benzene, 1-methyl-3-(1-methylethyl)-benzene, 2-butyl,1-octanol, decanal, limonene, and β-pinene. As in the PCA, we find that *C. immitis* RS and *C. posadasii* RMSCC3506 are clustering separately when grown as mycelia, partially driven by low abundances of VOCs from Cluster 1. Cluster 2 includes 16 VOCs that are present in spherule samples while generally absent or at a lower abundance in mycelia samples, and all of which are significantly different between lifecycles ($p < 0.05$ with Benjamini-Hochberg FDR correction). Cyclohexanone and 2,4,6-trimethlpryidine are two VOCs in this cluster that we were able to identify.


Strain
Population
Species
Lifecycle
Cluster 1
Cluster 2
Strain
RS
RMSCC2395
RMSCC3505
RMSCC2006
RMSCC2009
RMSCC2010
Silveira
B3221
B3222
GT-166
RMSCC2343
RMSCC3506
Media
Population
SDMX
SJV
AZ
TX/MX/SA
Species
C. immitis
C. posadasii
Lifecycle
Spherule
Mycelia
VOC Relative Abundance
5
0
−5

240

241 **Figure 5.** Hierarchical clustering analysis (HCA) of 72 fungal cultures and 6 media blanks
242 (columns) based on the relative abundance of 353 volatiles (rows). Clustering of fungi and blank
243 samples is using Pearson correlations, and clustering of volatiles is using Euclidean distance,
244 both with average linkage. Samples are color-coded by strain, population, species, and lifecycle,
245 as labeled in the legend; media blanks are in gray.

246

247 **DISCUSSION**

248 The genus *Coccidioides* is divided into two closely related and putatively allopatric species, with
249 a strong phylogeographic signal and population substructure within each species (19). However,
250 we find that the character of the *Coccidioides* volatilome is determined by lifecycle rather than
251 species. Within each species, the two lifecycles have a relatively small overlap in their shared
252 volatilome, with 27% and 31% shared for *C. posadasii* and *C. immitis,* respectively (Figure 1).
253 While there were no volatiles that were significantly different in relative abundance between *C.*
254 *posadasii* and *C. immitis*, we observed 43 statistically-significant differences in the VOCs
255 produced during the two lifecycles (Figure 2). This global difference between lifecycles is the
256 primary driver of separation we observed in multivariate analyses using PCA (Figure 4) and
257 HCA (Figure 5). These findings are in line with the large-scale transcriptional changes that occur
258 when *Coccidioides* spp. transitions from mycelial to spherule growth; between 20% and 40% of
259 the *Coccidioides* genes are differentially regulated during the shift between the environmental
260 and parasitic lifecycles (43-46). It has also been shown that the proteomes of the two lifecycles
261 are distinct, with statistically-significant differences between mycelia and spherule lifecycles
262 (47). In contrast to the significant differences in the volatilomes between lifecycles, we observed
263 no uniform differences between the volatilomes of *C. posadasii* and *C. immitis*. There is a high
264 degree of overlap (56 – 57%) between the species' volatilomes when comparing the same
265 lifecycle (Figure 1). This similarity is also visible in the PCA (Figures 4 and S1) and HCA
266 (Figure 5), as the volatilomes do not cluster by species even when we control for lifecycle.

267 Global similarities between *C. posadasii* and *C. immitis* have also been demonstrated at the
268 protein level (47, 48).
269
270 The lack of significant differences between the *C. posadasii* and *C. immitis* volatilomes is due, in
271 part, to large differences observed between strains of the same species; the volatilome highlights
272 some unique aspects of a few of the individual *Coccidioides* strains that we analyzed. For
273 example, RS is the type strain for *C. immitis*; however, it appears to be a metabolic outlier during
274 both lifecycles (Figure S1), but especially during mycelial growth (Figure 5). Additionally, the
275 mycelia lifecycle of *C. posadasii* RMSCC3506 appears to be a metabolic outlier compared to the
276 other strains grown as mycelia (Figures S1 and 5). These mycelia outliers appear to be driven, at
277 least in part, by the lack of volatiles that make up Cluster 1 in the HCA (Figure 5). None of the
278 56 VOCs found in Cluster 1, which are more abundant in mycelia compared to spherule cultures,
279 are detected in RMSCC3506 cultures, and only two are detected in RS mycelia cultures. These
280 two strains are also lacking VOCs 39 and 748, which were detected in the majority of strains for
281 both lifecycles, but absent from both lifecycles of RS and mycelia cultures of RMSCC3506
282 (Figure 3). Additionally, none of the 11 VOCs significantly more abundant in mycelia over
283 spherule cultures (Figure 2 and Table S1) are detected in RMSCC3506, and only two are
284 detected in RS. Beyond contributing to high levels of volatilome variance in *Coccidioides*, the
285 significance of the strain level differences relative to fungal biology or infection are not known.
286 Variation in virulence in murine models of coccidioidomycosis among strains has been observed,
287 but has not been tested in the context of species or with a mechanistic hypothesis to test (49). In
288 future studies of mouse model and human infections we plan to investigate correlations between
289 the *Coccidioides* volatilome and virulence and infection outcomes.

290

291 It should be noted that although the spherule and mycelia media had the same composition, we
292 detected different VOCs in the headspace of the blanks (Table S1, Figures 4 and 5). We interpret
293 these differences as the influence of the cultures on the blanks, rather than the influence of the
294 blank media on the cultures. Because the media blanks were incubated and processed in parallel
295 with their respective samples, we posit that the blank media absorbed volatiles from the culture
296 environment (e.g., from fungal spherule or mycelial cultures in shared incubators), which
297 introduced differences in their volatilomes. An analysis of fresh blank media (not incubated with
298 the cultures) would be required to test this hypothesis. Our data indicate that the spherule media
299 absorbed a greater abundance of compounds compared to the mycelia media (Table S1), which
300 may be due to the spherule lifecycle producing greater numbers and/or abundances of individual
301 compounds. Therefore, it is possible there are additional significant spherule volatiles that were
302 not identified within this study, since a VOC was defined as "detected" based on its relative
303 abundance versus media blanks. Future *in vitro* work needs to be mindful of volatile absorption
304 by the cultures and blanks within a shared space, such as an incubator.

305

306 The results from this study highlight two crucial factors that need to be considered in the
307 development and translation of volatilomics (and other functional genomics data) into
308 biomarkers. First, there can be a significant amount of intra-species variation, such that the
309 volatilome of a single strain is not representative of the species as a whole (17, 27, 50, 51).
310 Therefore, the analysis of multiple strains should be performed to identify a core volatilome, and
311 the more genetically and phenotypically diverse the species, the more strains will need to be
312 analyzed to determine the consensus set of volatiles produced by the majority of them. Second,

313 our data show that the growth phase of the organism has a strong influence on the volatilome,
314 and therefore using *in vitro* culture conditions that can most closely replicate the *in vivo*
315 environment and microbial growth phases may increase the likelihood of putative biomarker
316 translation from bench to bedside. In addition to controlling the growth phase of dimorphic
317 organisms, macronutrient, micronutrient, and oxygen availability should all be considered in the
318 development of *in vitro* model systems that replicate *in vivo* physiology of the pathogen (14, 17,
319 27, 30, 33, 36, 52, 53). Our next step in developing a Valley fever breath test is to translate *in*
320 *vitro* biomarkers for use with an *in vivo* mouse model. We observed 35 VOCs present in at least
321 67% of all spherule strains (Figure 3); as this is the parasitic lifecycle found within the host, we
322 hypothesize that the most highly conserved spherule VOCs have the greatest probability of
323 translating to Valley fever breath biomarkers.
324

325 **MATERIALS AND METHODS**

326 **Fungal Isolates and Growth Conditions**

327 *Isolates:* The *Coccidioides* isolates used in the study were obtained from human patients and are
328 listed in Table 1. All *Coccidioides* isolates were grown under BSL-3 containment, using
329 conditions that induce mycelial or spherule growth.
330

331 For mycelial growth, a 50 ml vented falcon tube containing 10 ml of RPMI media (filter
332 sterilized RPMI 1640, 10% fetal bovine serum) was inoculated with a 1 cm × 1 cm 2xGYE agar
333 plug for each strain. These plates were inoculated using 100 µl of glycerol stock, spread across
334 the plate, and cultured for 30°C for two weeks. Control RPMI media was inoculated with a plug
335 from sterile 2xGYE agar media. Each sample, including media control, was prepared in

336 triplicate. Cultures were grown on a shaking incubator at 150 rpm, 30°C for 96 h. For spherule
337 cultures, a 50 ml vented falcon tube containing 10 ml of RPMI media was inoculated to a final
338 concentration of 1.0×10^5 arthroconidia/ml in 1x phosphate-buffered saline (PBS).
339 Arthroconidia were grown and harvested as previously described (54). Strains RMSCC2343 and
340 RMSCC3505 did not produce enough conidia to achieve 1.0×10^5 arthroconidia/ml, and were
341 inoculated at 7.0×10^4 and 4.0×10^4 arthroconidia/ml, respectively. Control media was
342 inoculated with 1 ml of sterile 1xPBS. Cultures were grown on a shaking incubator at 150 rpm,
343 at 39°C in 10% CO_2 for 96 h. Mycelial and spherule cultures were spun at 12,000 × g at 4°C for
344 10 min to pellet the cells. The supernatant was removed and place in a Nanosep MF Centrifugal
345 Devices with Bio-Inert® Membrane 0.2 µm spin filter and centrifuged at 3,200 x g for 4 min.
346 The filtrate was stored at −80°C until volatile metabolomics analysis.
347
348 To ensure sterility of the filtrates for metabolomics analyses outside of a BSL-3 containment
349 facility, 10% of all sample filtrates were plated on 2xGYE and incubated at 30°C for 96 h to
350 ensure complete removal of viable pathogen particles. No growth was observed in any replicate.
351
352 **Volatile metabolomics analysis by SPME-GC×GC–TOFMS**
353 The *Coccidioides* spp. culture filtrates and media blanks were allowed to thaw at 4°C overnight,
354 and then 2 ml were transferred and sealed into sterilized 10 ml GC headspace vials with
355 PTFE/silicone septum screw caps. All samples were stored for up to 12 d at 4°C until analyzed.
356 Samples were randomized for analysis. Volatile metabolites sampling was performed by solid-
357 phase microextraction (SPME) using a Gerstel® Multipurpose Sampler directed by
358 Maestro® software. Volatile metabolite analysis was performed by two-dimensional gas

359 chromatography−time-of-flight mass spectrometry (GC×GC–TOFMS) using a LECO Pegasus
360 4D and Agilent 7890 GC (St. Joseph, MI). Chromatographic, mass spectrometric, and peak
361 detection parameters are provided in Table S3 (GC parameters). An external alkane standards
362 mixture (C_8 – C_{20}; Sigma-Aldrich, St. Louis, MO) was sampled multiple times for calculating
363 retention indices (RI). The injection, chromatographic, and mass spectrometric methods for
364 analyzing the alkane standards were the same as for the samples.
365
366 **Processing and analysis of chromatographic data**
367 Data collection, processing, and alignment were performed using ChromaTOF software version
368 4.71 with the Statistical Compare package (Leco Corp.), using the parameters listed in Table S3
369 (GC parameters).
370
371 Peaks were assigned a putative identification based on mass spectral similarity and RI data, and
372 the confidence of those identifications are indicated by assigning levels 1 – 4 (1 highest) (55).
373 Peaks with a level 1 ID were identified based on mass spectral and retention index matches with
374 external standards. Peaks with a level 2 ID were identified based on ≥ 800 mass spectral match
375 by a forward search of the NIST 2011 library and RI that are consistent with the midpolar Rxi-
376 624Sil stationary phase, as previously described (50), but using an RI range of 0 – 43%
377 (empirically determined by comparing the Rxi-624Sil RIs for Grob mix standards to published
378 polar and non-polar values). Level 1, 2, and 3 compounds were assigned to chemical functional
379 groups based upon characteristic mass spectral fragmentation patterns and second dimension
380 retention times, as previously described (51). Level 4 compounds have mass spectral matches <

381 600 or retention indices that do not match previously published values, and are reported as
382 unknowns.
383
384 **Data post processing and statistical analyses**
385 The data post processing steps are depicted in Figure S2. Before statistical analyses, compounds
386 eluting prior to 358 s (acetone retention time) and siloxanes (*i.e.,* chromatographic artifacts) were
387 removed from the peak table. Peaks that were present in only one of the three biological
388 replicates were imputed to zero for that sample, while missing values for peaks that were present
389 in two out of three biological replicates were imputed to half of the minimum value across all
390 biological replicates. The relative abundances of compounds across chromatograms were
391 normalized using probabilistic quotient normalization (PQN) (56) in R version 3.4.3. The data
392 were $\log_{10}$ transformed, and intraclass correlation coefficients (ICCs) were calculated using R
393 *ICC* package version 2.3.0, and peaks with an ICC < 0.75 were not further processed. Analytes
394 were retained for further analysis if they were at least two-fold more abundant in any
395 *Coccidioides* culture compared to media controls, and were considered to have been detected in
396 that culture. Principal component analysis was performed using R *factoextra* package version
397 1.0.5 with the biological replicates as observations and the absolute peak intensities (mean-
398 centered and scaled to unit variance) as variables. The relatedness of samples based on their
399 volatile metabolomes were assessed using hierarchical clustering analysis on the Pearson's
400 correlation between isolates and Euclidean distance between volatiles using R *pheatmap* package
401 version 1.0.12. Geometric means of the biological replicates were calculated and used to
402 determine the statistical difference in abundance between lifecycles or species using a Mann-
403 Whitney *U*-test and Benjamini and Hochberg false discovery rate correction with $\alpha = 0.05$, using

404 R *stats* package version 3.5.3. The variation in abundance of volatiles significantly different
405 between lifecycle was calculated by dividing mean mycelia peak abundance by mean spherule
406 peak abundance.
407
408 **Data availability**
409 Metabolomic data (chemical feature peak areas and retention time information)
410 included in this study are available at the NIH Common Fund's National Metabolomics Data
411 Repository (NMDR) website, the Metabolomics Workbench, at
412 www.metabolomicsworkbench.org, where it has been assigned project ID PR000### and study
413 ID ST00### (https://doi.org/####).
414
415 **CONCLUSION**
416 The *Coccidioides* volatilome is strongly influenced by the environmental versus parasitic
417 lifecycles, while the two species, *C. posadasii* and *C. immitis*, are indistinguishable by their
418 volatile profiles, in part due to high strain to strain variability within the species. We identified
419 35 VOCs produced by the majority of the *Coccidioides* strains when grown as spherules, the
420 parasitic form of the fungus found in Valley fever lung infections. Our results show that as future
421 efforts are made to develop biomarkers for *Coccidioides* and other mycoses caused by dimorphic
422 fungi, it is vitally important to control for fungal lifecycle and sample multiple strains of the
423 pathogen to identify volatiles that are more highly conserved within the infectious genus or
424 species.
425
426

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431 We declare that the research was conducted in the absence of any commercial or financial
432 relationships that could be construed as a potential conflict of interest.

433

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