A novel virulence phenotype rapidly assesses *Candida* fungal pathogenesis in healthy and immunocompromised *Caenorhabditis elegans* hosts

Dorian J. Feistel, Rema Elmostafa, Nancy Nguyen, McKenna Penley, Levi Morran, and Meleah A. Hickman*

Running title: Fungal infection reduces multiple measures of host fitness

Department of Biology, Emory University
*corresponding author

Abstract

The yeast *Candida albicans* is an opportunistic pathogen of humans, meaning that despite commensal interactions with its host, it can transition to a harmful pathogen. While *C. albicans* is the predominant species isolated in the human mycobiome and implicated in fungal infection, infections due to non-albicans *Candida* species are rapidly rising. Studying the factors that contribute to virulence is often challenging and frequently depends on many contexts including host immune status and pathogen genetic background. Here, we utilize the nematode *Caenorhabditis elegans* as a perspicuous and efficient model host system to study fungal infections of *Candida* pathogens. We find that in addition to reducing lifetime host survival, exposure to *C. albicans* results in delayed reproduction, which significantly reduced lineage growth over multiple generations. Furthermore, we assessed fungal pathogen virulence in *C. elegans* hosts compromised for innate immune function and detected increased early mortality, reduced brood sizes and delayed reproduction relative to infected healthy hosts. Importantly, by assessing virulence in both healthy and immunocompromised host backgrounds we reveal the pathogen potential in non-*albicans Candida* species. Taken together, we present a novel lineage growth assay to measure reduction in host fitness associated with fungal infection and demonstrate significant interactions between pathogen and host immune function that contribute to virulence.

Importance

Opportunistic pathogens are commensals capable of causing disease and are serious threats to human health. It is critical to understand the mechanisms and host contexts under which opportunistic pathogens become virulent. In this work, we present a novel assay to quickly and quantitatively measure pathogen virulence in healthy and immunocompromised hosts. We found that *Candida* species, the most prominent fungal opportunistic pathogens of humans, decrease host fitness by reducing survival and impacting host reproduction. Most importantly, by measuring virulence in hosts that have intact or compromised immune function, we can reveal the pathogenic potential of opportunistic fungal pathogens.

63 **Introduction**

64 *Candida* species are commensals of the human gastrointestinal microbiota and
65 various other niches in the human body [1]. Despite their commensal existence in many
66 humans, *Candida* species also account for the most fungal infections in the world and
67 are the fourth most prevalent cause of all nosocomial blood stream infections [2,3]. The
68 severity of fungal infection is often dependent on the immune status of the host, with
69 superficial infections occurring in healthy individuals and bloodstream infections in
70 immunocompromised hosts [4-8]. The majority of these infections are associated with
71 *Candida albicans,* however other non-*albicans Candida* species are increasingly
72 becoming implicated in fungal infections [9,10].

73 Several host models have been employed to better understand fungal infection
74 and disease progression. These experimental systems include mouse, zebrafish,
75 nematode, wax moth and human *ex vivo* models have been specifically developed to
76 study *Candida* infections [11]. Most often, virulence phenotypes are limited to survival
77 outcomes or tissue-specific fungal burden [12], the latter of which requires animal
78 sacrifice and cannot address the long-term consequence of fungal disease.
79 Furthermore, animal mortality and sacrifice, particularly in vertebrate models, constrain
80 the number of infected individuals to study and thus reduce the quantitative robustness
81 of these experiments. Nonetheless, these studies provide valuable insight into *Candida*
82 infection and disease and have uncovered important roles for innate immune function of
83 hosts [13-15], specific *C. albicans* genes regulating virulence [16,17] and differences
84 between genetic backgrounds within and between *Candida* species [18].

85 Other major limitations for most studies of *Candida* infection virulence include the
86 time-intensive nature of tracking virulence phenotypes in vertebrate systems such as
87 mice, the genetic intractability of zebrafish and wax moth larvae and the context-
88 independent nature of *ex vivo* models [4]. As such, most studies focus specifically on *C.*
89 *albicans* virulence while many non-albicans *Candida* species are evaluated to a lesser
90 degree. The nematode *Caenorhabditis elegans* has been developed as a model for
91 *Candida* infection to overcome these limitations [19,20] and to screen compounds for
92 antifungal activity [21]. *C. elegans* has proven useful for studying host-microbe
93 interactions [22] because many fungal and bacterial pathogens that cause illness in

94 humans also cause disease in *C. elegans* [23]. Infecting *C. elegans* is relatively simple
95 and often done by replacing or incorporating its standard laboratory food *E. coli* with a
96 desired pathogen which colonizes the gut and causes disease [23]. Furthermore, the
97 innate immune system in *C. elegans* includes the *SEK-1* gene which encodes a MAP
98 Kinase Kinase (MAPKK) that is homologous to the MKK3/6 and MKK4 family of
99 mammalian MAPKKs and activates the *C. elegans* p38 MAP kinase ortholog [24]. This
100 pathway has been suggested to be an ancient and conserved component of *C. elegans'*
101 immune response to pathogens [25]. As such, the *sek-1* mutation increases
102 susceptibility to microbial colonization [26], including infection with many *Candida*
103 species [27] and is necessary to induce the appropriate antifungal immune defense [28].
104 In addition to utilizing innate immune system mutants, most studies measure host
105 mortality in temperature sensitive sterile mutants [17,21,29,30] to more easily track
106 mortality in founder populations since *C. elegans* has a rapid lifecycle and large brood
107 sizes [31,32]. The effects on host reproduction are often overlooked in favor of
108 examination of mortality rates when assessing a pathogen's virulence [33]. Yet, it is
109 important to remember that virulence can be broadly measured as any reduction in host
110 fitness resulting from interactions between a pathogen and its host [34-36]. In this work,
111 we assessed virulence of *Candida* species in *C. elegans* hosts using a novel measure
112 of host fitness that incorporated both host survival and fecundity. We found that fungal
113 pathogens reduced host fitness by delaying reproduction, resulting in long-term
114 consequences for population growth in both healthy and immunocompromised host
115 backgrounds. Using this novel measure, we characterized virulence phenotypes for
116 three non-*albicans Candida* species; *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis* in
117 both healthy and immunocompromised *C. elegans* hosts. Our studies demonstrate that
118 differences in virulence can be identified between pathogen species and that
119 pathogenic potential is often revealed when host immune function is diminished.
120

121 **Methods**

122 *Strains and media*

123 For this study, the fungal pathogens *C. albicans* (SC5314 [37]), *C. dubliniensis*
124 (Wu284 [38]), *C. tropicalis* (ATCC 22109), and *C. parapsilosis* (ATCC 22109) strains

125 were used. *C. elegans* N2 Bristol (WT) and a *sek-1* mutant derivative [24] were used to
126 test host survival, fecundity and population growth. *C. elegans* populations were
127 maintained at 20°C on 100mm petri dishes with 25 mL of lite nematode growth medium
128 (NGM, US Biological) with *E. coli* OP50 as a food source. Nematodes were transferred
129 to a newly seeded *E. coli* plate every 3-4 days. For survival, fecundity and population
130 growth assays, NGM was supplemented with 0.08 g/L uridine, 0.08 g/L histidine, and
131 0.04 g/L adenine to facilitate growth of auxotrophic *C. albicans* strains and 0.2 g/L
132 streptomycin sulfate to inhibit *E. coli* overgrowth so fungal strains could proliferate.
133
134 *Seeding NGM plates for survival, fecundity, and population growth assays*
135 *Candida* strains and *E. coli* OP50 strains were inoculated in 3 mL of YPD or 5 mL
136 of LB, respectively, and cultured at 30°C for 1-2 days. *Candida* culture densities were
137 measured with a spectrophotometer and diluted to a final volume of 3.0 OD_{600} per mL
138 (~6 x 10^7 cells per ml). *E. coli* cultures were pelleted and washed twice with 1 mL of
139 ddH_2O. The supernatant was removed and the pellet was centrifuged for 60 sec at
140 maximum to remove any excess liquid. The pellet was weighed and suspended with
141 sterilized water to a final volume of 200 mg/mL. A mix of 6.25 μL *E. coli*, 1.25 μL
142 *Candida* was brought to a final volume of 50 μL with ddH_2O. The entire 50 μL was
143 spotted onto the center of a 35mm supplemented-NGM Lite agar plate and incubated at
144 room temperature overnight before addition of eggs or transferring nematode. *E. coli*
145 OP50 was used as a control at the same concentration described above.
146
147 *Egg preparation and synchronization for survival, fecundity and population growth*
148 *assays*
149 For survival, fecundity and population growth assays, approximately 100
150 nematodes at the L3/L4 stage were transferred to a 100mm NGM plate seeded with *E.*
151 *coli* OP50 and maintained at 20°C for 2-3 days prior to the start of an experiment. On
152 the first day of an experiment, these NGM plates were washed with M9 buffer and
153 contents (live nematodes and eggs) transferred to 15 mL conical tube and pelleted by
154 centrifugation (2 min at 1200 rpm). The pellet was re-suspended in a 1:4 bleach (5.25%)
155 solution and transferred to a micro-centrifuge tube. The suspension was mixed via

156 inversion for 90-120 sec and subsequently centrifuged (30 sec at 1500 rpm). The pellet
157 was washed with 1 mL M9 buffer three consecutive times to remove excess bleach
158 solution and brought to a final suspension with 500 μL M9 buffer. To determine the
159 concentration of eggs, 10 μL was placed on a concaved slide and eggs counted and the
160 egg suspension was diluted with M9 to a final concentration of 10 eggs/μL. All assays
161 were treated equally on the first day (Day 0) by adding roughly 100 eggs to a treatment
162 or *E. coli* plate (described above).
163
164 *Survival assays*
165 This experimental procedure is a modified version of [39]. Briefly, 72 h after
166 adding ~50-75 nematode eggs to a plate, 40 adult nematodes were randomly selected
167 and transferred to newly seeded plates with the same concentration of food as
168 described above and incubated at 20°C. Every other day nematodes were transferred to
169 freshly seeded plates until all nematode replicate populations went extinct. The number
170 of living, dead, and censored worms were scored daily. Each survival assay was
171 performed at least three independent times.
172
173 *Fecundity assays*
174 For both fecundity and lineage expansion assays, 48 h after adding nematode
175 eggs to a plate, a single L4 reproductively immature hermaphroditic nematode was
176 randomly selected and transferred (6-10 independent biological replicates per treatment
177 per block) to a newly seeded 35 mm petri plate containing either a treatment of *C.*
178 *albicans* and *E. coli* or *E. coli* alone (described above) and incubated at 20°C.
179 Nematodes were transferred to freshly seeded plates every 24 h for seven consecutive
180 days. Eggs remained undisturbed on the plate and were incubated at 20°C for an
181 additional 24 h to provide enough time to hatch, at which point the number of viable
182 progeny per day was scored. Nematodes that died during the assay were scored as
183 dead at the time of transfer. Nematodes that crawled off the plate or were otherwise
184 unaccountable for were considered censored and excluded from the analysis.
185
186 *Lineage Expansion Assay*

187 A single L4 reproductively immature hermaphroditic nematode was randomly
188 selected and transferred (6 biological replicates per treatment) to a freshly seeded 100
189 mm treatment or *E. coli* plate (described above) containing a 6-fold increase in food.
190 Five days later, each plate was washed with M9 buffer until the majority of nematodes
191 were displaced and subsequently transferred to 15mL conical tubes. Tubes were placed
192 at 4°C for 1h to allow the nematodes to settle at the bottom. All tubes were
193 concentrated to a final volume of 10mL. Six, 20 μL samples were taken from each
194 population and counted.
195 *Statistical analyses*
196 All statistical analysis were performed with GraphPad Prism. Data sets were
197 tested for normality using the D'Agostino & Pearson omnibus normality test. For
198 comparisons across groups, one-way ANOVAs and post-hoc Tukey multiple
199 comparison tests or Kruskal-Wallis and Dunn's multiple comparison tests were
200 performed.
201
202 **Results**
203 *Exposure to C. albicans reduces multiple measures of C. elegans fitness*
204 Pathogens affect host fitness by decreasing lifespan and/or reducing fecundity.
205 To determine how *C. albicans* impacts *C. elegans* host fitness, we first measured
206 nematode lifespan in the presence or absence of *C. albicans*. We observed a significant
207 reduction in survival when *C. elegans* was reared on *C. albicans* compared to when it
208 was reared in its absence (Figure 1, $p < 0.0001$ Log-rank test), consistent with
209 previously published results [39]. 50% mortality was reached in eight days in nematode
210 populations exposed to *C. albicans* compared to 16 days in unexposed nematode
211 populations, indicating that *C. elegans* lifespan is substantially decreased by exposure
212 to *C. albicans*.
213 While overall survival was reduced, nematode death was rarely observed earlier
214 than 5 days post-infection. Importantly, adult nematodes produce the majority of their
215 offspring before *C. albicans* infection reduces rates of survival. Given this information
216 and that fecundity is a key component of fitness from an evolutionary perspective, we
217 investigated whether *C. albicans* negatively impacts *C. elegans* reproduction. To test

218 this, we counted the total number of viable progeny (i.e. brood size) produced in the first
219 seven days of reproductive maturity from individual nematodes that were either
220 unexposed or exposed to either heat-killed or live *C. albicans*. As shown in Figure 2A,
221 exposure to *C. albicans* reduced the average number of viable progeny (253±7)
222 compared to the unexposed control (285±4; $p < 0.0002$, Kruskal-Wallis test) by ~11%.
223 While statistically significant, the cost of *C. albicans* toward host brood size is modest.
224 Furthermore, the brood size of nematodes exposed to heat-killed *C. albicans* (HK) was
225 not detectably different from uninfected or live *C. albicans* treatments (Figure 2A).
226 Intriguingly, we identified a significant delay in reproductive timing when we
227 measured the number of progeny produced every 24 h during the host reproductive
228 window. We observed a substantial reduction in reproduction on Day 3 in nematodes
229 exposed to *C. albicans* (103±4) relative to unexposed hosts (187±3) or hosts associated
230 with heat-killed *C. albicans* (Figure 2B; $p < 0.0001$ Kruskal-Wallis test) and increased
231 progeny produced on Days 4-6 in nematodes exposed to *C. albicans* (Figure 2B). We
232 calculated the total fraction of reproduction occurring in this 'late' window (Days 4-6) and
233 identified a significant increase in the progeny produced late in *C. elegans* exposed to
234 *C. albicans* compared to unexposed or heat-killed controls (Figure 2C; p=0.0001,
235 Kruskal-Wallis test). Taken together, our data indicate that *C. albicans* severely delays
236 and reduces reproduction in addition to impacting overall survival in *C. elegans.*
237 To investigate whether this reproductive delay observed in nematodes exposed
238 to *C. albicans* had any long-term consequences, we took advantage that *C. elegans* are
239 hermaphrodites and measured the total population produced from a single founder
240 worm over multiple generations through lineage expansion assays. We find that the
241 median population produced within 7 days from a single uninfected nematode is
242 ~30,250 F1 and F2 progeny (Figure 2D). Lineage expansion is significantly impacted in
243 nematodes exposed to live *C. albicans*, with a median population size of ~20,208 F1
244 and F2 progeny, a reduction of ~33% compared to uninfected and heat-killed control
245 treatments (Figure 2D, $p < 0.0001$ one-way ANOVA). We conclude that the delay in
246 reproduction, coupled with the reduced brood size resulting from exposure to *C.*
247 *albicans* severely impacts *C. elegans* evolutionary fitness.
248

249 *Immunocompromised hosts are susceptible to fungal infection*

250 A leading host-related risk factor for invasive candidiasis is compromised
251 immune function [40]. Given our novel results regarding fecundity in healthy *C. elegans*
252 hosts, we were curious how exposure to *C. albicans* impacts nematode hosts with
253 compromised immune function. To address this point, we utilized a *C. elegans* strain
254 carrying a mutation in *SEK*-1, a well-conserved MAP kinase involved in the innate
255 immune signaling cascade [24,26] and antifungal response [28]. Unlike healthy hosts,
256 immunocompromised hosts show high rates of early (within 7 days) mortality in both
257 uninfected and *C. albicans* treatments (Figure S1). *C. albicans* exposure reduced
258 average brood size by nearly 50% (78±6) in immunocompromised hosts compared to
259 uninfected (151±5) and heat-killed (127±13) controls (Figure 3A, $p < 0.0001$, Kruskal-
260 Wallis test). It is important to note that in all treatments (unexposed, heat-killed, and live
261 *C. albicans*), immunocompromised hosts produced significantly less progeny than
262 healthy hosts (Table S1), but the largest reductions occur in immunocompromised hosts
263 are exposed to live *C. albicans*.

264 One explanation for the overall reduction in average brood size is the increased
265 early mortality we observe in immunocompromised hosts. To address this possibility,
266 we re-analyzed brood size only from hosts that survived past Day 3 and found that while
267 this did slightly increase average brood size across all treatments, it was not significant
268 between analyses ($p=0.2356$ two-tailed t-test) and exposure to *C. albicans* still
269 significantly reduced average brood size (94±6) relative to uninfected (163±5) and heat-
270 killed controls (141±9) (Figure S2, $p < 0.0001$ Kruskal-Wallis test). For hosts that
271 survived past three days, we also detected a significant delay in reproductive timing
272 upon exposure to *C. albicans*, with nearly 45% of all reproduction occurring in this late
273 window (Figure 3C), similar to what we observe in healthy hosts (Table S1).

274 We predicted that lineage expansion in immunocompromised hosts exposed to
275 *C. albicans* would be more dramatically impacted relative to wild-type nematodes
276 exposed to *C. albicans*, given the reproductive delays coupled with the overall reduced
277 brood sizes and increased mortality observed. Indeed, we determined that the median
278 population size of *C. albicans* exposed nematodes was ~11,000 compared to ~17,000
279 and 19,500 in heat-killed and uninfected controls, respectively (Figure 3C). The overall

280 reduction in population size resulting from *C. albicans* exposure is ~43% in
281 immunocompromised hosts and is significantly greater than the ~32% reduction in
282 population size in healthy hosts (Figure 4 and Table S1). Taken together, our results
283 indicate that immunocompromised hosts are more susceptible to fungal infection than
284 healthy wild-type hosts.
285
286 *Host immune status reveals pathogenic potential in other Candida species*
287 While *C. albicans* is the predominant fungal agent in invasive candidiasis, non-
288 *albicans Candida* species (NACs) are estimated to account for 35-65% of
289 candidaemia [41]. By evaluating NACs in both healthy and immunocompromised hosts,
290 we can begin to assess their pathogenic potential, and thus extend the utility of *C.*
291 *elegans* as a model host system for detecting virulence in *Candida* species. Here, we
292 leveraged our simple, yet robust, lineage expansion assays to measure the impact of
293 three additional *Candida* species: *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* on
294 host fitness for healthy and immunocompromised *C. elegans*. All three non-*albicans*
295 *Candida* species significantly reduced the population size of both healthy and
296 immunocompromised hosts compared to uninfected controls (black asterisks, Figure S3
297 and Table S1), and sometimes were also significantly different than population sizes of
298 hosts exposed to *C. albicans* (red asterisks, Figure S3).
299 We next wanted to determine if there was a significant interaction between
300 *Candida* species and host immune function. To test this, we evaluated the population
301 size of infected nematodes relative to uninfected for healthy (Figure 4; black bars) and
302 immunocompromised (Figure 4; green bars) host backgrounds, since
303 immunocompromised hosts have significantly smaller populations even when
304 uninfected (Figure 2 and S4). There are significant differences among *Candida* species
305 and nematode host backgrounds ($p < 0.001$, two-way ANOVA; $p < 0.001$ between
306 pathogens, hosts, and their interaction). In healthy hosts, *C. albicans* and *C. tropicalis*
307 are the most virulent, with 32-35% reductions in total population size, whereas *C.*
308 *parapsilosis* and *C. dublineinsis* result in more modest population reductions (17-20%).
309 However, in immunocompromised hosts, *C. parapsilosis* is one of the most virulent, with
310 nearly a 67% reduction in population size. *C. albicans* and *C. tropicalis* are also highly

311 virulent in immunocompromised hosts and all three of these species more severely
312 impact immunocompromised hosts compared to healthy hosts. In contrast, *C.*
313 *dublineinsis* is the least virulent, with no significant differences observed between the
314 relative population sizes of healthy and immunocompromised hosts (Figure 4). Given
315 the virulence phenotypes of these different yeasts, our results suggest that fungal
316 virulence depends not only on the species of pathogen but also on the immune status of
317 the host.
318
319 **Discussion**
320 Here we utilized a *C. elegans* experimental host system to identify novel
321 measures of host fitness associated with fungal infection. By tracking the number of
322 progeny produced per day, we can quickly and quantitatively assess three aspects of
323 host fitness: early mortality, total viable offspring produced and reproductive timing. We
324 show that exposure to *Candia albicans* in healthy *C. elegans* hosts not only reduces
325 survival (Figure 1), it modestly reduces total progeny produced, and dramatically delays
326 reproductive timing (Figure 2). This reproductive delay has long-term consequences for
327 lineage expansion, as single founder nematodes exposed to *C. albicans* reduces its
328 population growth by ~30% (Figure 2D). We found similar delays in reproductive timing
329 in immunocompromised hosts exposed to *C. albicans*, although immunocompromised
330 hosts are more susceptible to infection and have higher incidence of early mortality and
331 smaller brood sizes compared to healthy hosts (Figure 3). Importantly, the delayed
332 reproduction phenotype in infected *C. elegans* is easily ascertained, highly quantitative
333 and reproducible and can be used as a screening method to detect relatively small
334 differences in virulence across *Candida* strains or other fungal species. Furthermore, by
335 utilizing different host contexts, this assay revealed the pathogenic potential of other
336 important, yet understudied, *Candida* species (Figure 4), including *C. parapsilosis*,
337 which has dramatically higher levels of virulence in immunocompromised hosts
338 compared to healthy hosts.
339 The reductions in host fitness associated with *C. albicans* and other *Candida*
340 species indicate that we have established a bona fide model of fungal infection.
341 Importantly, these reductions in host fitness cannot be attributed to *Candida* species

342 being a sub-optimal food source or that the hosts are starving because we deliver the
343 pathogen with *E. coli*, the standard food source for *C. elegans*. Further, we do not
344 detect any host larval development into dauer, an alternative lifestage in response to
345 starvation and overpopulation [32]. While pathogen avoidance is a common defense
346 strategy for *C. elegans* [42], we censored any worms that have crawled off the plate or
347 have disappeared during the course of our experiment and removed them from our data
348 analysis. Furthermore, the reductions in host fitness depend on ingesting live *C.*
349 *albicans*, as heat-killed treatments do not cause significant reductions in total brood
350 size, reproductive timing, or lineage expansion (Figure 2, 'HK'). Microscopic analysis
351 reveals that fungal cells inhabit the gut of *C. elegans* ([39] and data not shown) and we
352 can extract viable host-associated fungal cells (data not shown). Taken together, these
353 data support a model of fungal infection in the host *C. elegans*.
354 The host reproductive delay we observed in *C. elegans* upon fungal infection is a
355 robust, highly quantitative measure of virulence that makes it amenable to screen a
356 variety of host and pathogen genetic backgrounds that has been challenging in
357 mammalian and insect models. *C. elegans* is easily maintained in the lab, has a short
358 lifecycle that generates a large number of progeny, and has been a fundamental model
359 genetic organism [32]. Previous work has shown that upon exposure to *C. albicans, C.*
360 *elegans* have a transcriptional response consistent with that of infection [28]. In this
361 work, we utilized a mutant *C. elegans* strain in which *SEK-1*, an important MAPK in
362 innate immunity and whose homologs include the MKK3/6 and MKK4 family of
363 mammalian MAPKKs, to determine the severity of fungal infection in
364 immunocompromised hosts. We demonstrate that deficiencies in innate immune
365 function result in hosts with high susceptibility to fungal infection and in the future can
366 extend this analysis for many other host backgrounds that are mutant for immune
367 function.
368 Previous studies using *C. elegans* as a host for fungal infection have described
369 additional phenotypes beyond reduced survival, including a deformed anal region (DAR)
370 [39,43]. It is possible that DAR may be contributing to the delayed reproductive
371 phenotype we observe in our experiments, however, the frequency of DAR phenotypes
372 was low and we did not observe any detectable differences between hosts exposed to

373 *C. albicans* and hosts unexposed (data not shown), nor did we observe dramatic
374 reductions in total number of viable offspring produced between these two groups of
375 hosts (Figure 2A. While this deformity is a result of a local defense reaction of the worm
376 due to extracellular signal-regulated kinase activation of the innate immunity MAKK
377 cascade, it is a distinct marker from delayed reproduction for infection [44]. Wildtype
378 reproductive timing utilizes the highly conserved DAF-2 DAF-16 insulin-like signaling
379 pathway [23], but has also been implicated in innate immunity and bacterial infection
380 [45] and is potentially disrupted in *C. elegans* hosts infected with *Candida*.
381 Impacts on host reproduction have often been overlooked in studies regarding
382 *Candida* virulence, yet host reproduction is an important evolutionary measure of an
383 organism's fitness. Not only can we utilize these fecundity assays to assess the roles of
384 host immune function on fungal pathogenesis, we also readily screen diverse pathogen
385 strain backgrounds and species. A large-scale, international 10-year study identified 31
386 species of *Candida* associated with clinical samples. While *C. albicans* is still the most
387 prevalent, its isolation from clinical isolates is decreasing with corresponding increases
388 isolation of *C. glabrata, C. tropicalis* and *C. parapsilosis* [46]. Despite its close
389 evolutionary relationship with *C. albicans*, *C. dubliniensis* does not seem to be highly
390 pathogenic [47]. However, other non-*albicans Candida* species have been increasingly
391 implicated in fungal infections of humans [48]. For example, *C. tropicalis* and *C.*
392 *parapsilosis* are common fungal species isolated in patients with candidemia (7 - 48%
393 and 11 - 27% respectively and depending on geographic region), candiduria (8 – 44%
394 and 0.5 – 11% respectively, and oral candidosis (5 – 13% and 7 – 15%, respectively)
395 ([48] and references therein). Despite the increasing incidence of non-*albicans Candida*
396 infections [46], experimental studies using these pathogens remains limited. Here, we
397 analyzed the virulence phenotypes of three non-albicans *Candida* species, *C.*
398 *dubliniensis*, *C. tropicalis* and *C. parapsilosis* in both healthy and immunocompromised
399 host backgrounds. In healthy *C. elegans* hosts, *C. parapsilosis* is the least virulent
400 compared to *C. albicans* and *C. tropicalis* and *C. dublineinsis* has intermediate level of
401 virulence (Figure 4). For all species except for *C. dubliniensis,* virulence is more severe
402 in immunocompromised hosts and is particularly striking in *C. parapsilosis*, which is the
403 most virulent in hosts with compromised immune function. Therefore, the *C. elegans*

404 fecundity assays we developed in this study can rapidly reveal pathogenic potential by
405 assessing virulence in multiple host genetic backgrounds and across pathogens in a
406 highly quantitative and robust manner.
407
408 Literature Cited
409

410 1. Underhill, D. M.; Iliev, I. D. The mycobiota: interactions between commensal fungi
411 and the host immune system. *Nature reviews. Immunology* **2014**, *14*, 405–416.
412 2. Pappas, P. G.; Lionakis, M. S.; Arendrup, M. C.; Ostrosky-Zeichner, L.; Kullberg, B. J.
413 Invasive candidiasis. *Nat Rev Dis Primers* **2018**, *4*, 18026.
414 3. Edmond, M. B.; Wallace, S. E.; McClish, D. K.; Pfaller, M. A.; Jones, R. N.; Wenzel,
415 R. P. Nosocomial bloodstream infections in United States hospitals: a three-year
416 analysis. *Clin Infect Dis* **1999**, *29*, 239–244.
417 4. MacCallum, D. M. Hosting infection: experimental models to assay Candida
418 virulence. *Int J Microbiol* **2012**, *2012*, 363764–12.
419 5. Pfaller, M. A. Nosocomial candidiasis: emerging species, reservoirs, and modes of
420 transmission. *Clin Infect Dis* **1996**, *22 Suppl 2*, S89–94.
421 6. Coleman, D. C.; Sullivan, D. J.; Bennett, D. E.; Moran, G. P.; Barry, H. J.; Shanley, D.
422 B. Candidiasis: the emergence of a novel species, Candida dubliniensis. *AIDS* **1997**,
423 *11*, 557–567.
424 7. Kontoyiannis, D. P.; Vaziri, I.; Hanna, H. A.; Boktour, M.; Thornby, J.; Hachem, R.;
425 Bodey, G. P.; Raad, I. I. Risk Factors for Candida tropicalis fungemia in patients with
426 cancer. *Clin Infect Dis* **2001**, *33*, 1676–1681.
427 8. Clark, T. A.; Slavinski, S. A.; Morgan, J.; Lott, T.; Arthington-Skaggs, B. A.; Brandt, M.
428 E.; Webb, R. M.; Currier, M.; Flowers, R. H.; Fridkin, S. K.; Hajjeh, R. A.
429 Epidemiologic and molecular characterization of an outbreak of Candida parapsilosis
430 bloodstream infections in a community hospital. *J. Clin. Microbiol.* **2004**, *42*, 4468–
431 4472.
432 9. Pfaller, M. A.; Diekema, D. J.; Jones, R. N.; Sader, H. S.; Fluit, A. C.; Hollis, R. J.;
433 Messer, S. A.; SENTRY Participant Group International surveillance of bloodstream
434 infections due to Candida species: frequency of occurrence and in vitro

435 susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected
436 from 1997 through 1999 in the SENTRY antimicrobial surveillance program. *J. Clin.*
437 *Microbiol.* **2001**, *39*, 3254–3259.
438 10. Hajjeh, R. A.; Sofair, A. N.; Harrison, L. H.; Lyon, G. M.; Arthington-Skaggs, B. A.;
439 Mirza, S. A.; Phelan, M.; Morgan, J.; Lee-Yang, W.; Ciblak, M. A.; Benjamin, L. E.;
440 Sanza, L. T.; Huie, S.; Yeo, S. F.; Brandt, M. E.; Warnock, D. W. Incidence of
441 bloodstream infections due to Candida species and in vitro susceptibilities of isolates
442 collected from 1998 to 2000 in a population-based active surveillance program. *J.*
443 *Clin. Microbiol.* **2004**, *42*, 1519–1527.
444 11. Harwood, C. G.; Rao, R. P. Host pathogen relations: exploring animal models for
445 fungal pathogens. *Pathogens* **2014**, *3*, 549–562.
446 12. Conti, H. R.; Huppler, A. R.; Whibley, N.; Gaffen, S. L. Animal models for
447 candidiasis. *Curr Protoc Immunol* **2014**, *105*, 19.6.1–17.
448 13. Verma, A.; Gaffen, S. L.; Swidergall, M. Innate Immunity to Mucosal Candida
449 Infections. *J Fungi (Basel)* **2017**, *3*, 60.
450 14. Naglik, J. R.; König, A.; Hube, B.; Gaffen, S. L. Candida albicans-epithelial
451 interactions and induction of mucosal innate immunity. *Curr Opin Microbiol* **2017**, *40*,
452 104–112.
453 15. Richardson, J. P.; Moyes, D. L.; Ho, J.; Naglik, J. R. Candida innate immunity at the
454 mucosa. *Seminars in Cell & Developmental Biology* **2018**.
455 16. Lu, Y.; Su, C.; Solis, N. V.; Filler, S. G.; Liu, H. Synergistic regulation of hyphal
456 elongation by hypoxia, CO(2), and nutrient conditions controls the virulence of
457 Candida albicans. *Cell Host Microbe* **2013**, *14*, 499–509.
458 17. Pukkila-Worley, R.; Peleg, A. Y.; Tampakakis, E.; Mylonakis, E. Candida albicans
459 hyphal formation and virulence assessed using a Caenorhabditis elegans infection
460 model. *Eukaryotic Cell* **2009**, *8*, 1750–1758.
461 18. Hirakawa, M. P.; Martinez, D. A.; Sakthikumar, S.; Anderson, M. Z.; Berlin, A.; Gujja,
462 S.; Zeng, Q.; Zisson, E.; Wang, J. M.; Greenberg, J. M.; Berman, J.; Bennett, R. J.;
463 Cuomo, C. A. Genetic and phenotypic intra-species variation in Candida albicans.
464 *Genome Res* **2015**, *25*, 413–425.

465 19. Powell, J. R.; Ausubel, F. M. Models of Caenorhabditis elegans infection by
466 bacterial and fungal pathogens. *Methods Mol Biol* **2008**, *415*, 403–427.
467 20. Aballay, A.; Ausubel, F. M. Caenorhabditis elegans as a host for the study of host-
468 pathogen interactions. *Curr Opin Microbiol* **2002**, *5*, 97–101.
469 21. Breger, J.; Fuchs, B. B.; Aperis, G.; Moy, T. I.; Ausubel, F. M.; Mylonakis, E.
470 Antifungal chemical compounds identified using a C. elegans pathogenicity assay.
471 *PLoS Pathog* **2007**, *3*, e18.
472 22. Sifri, C. D.; Begun, J.; Ausubel, F. M. The worm has turned--microbial virulence
473 modeled in Caenorhabditis elegans. *Trends Microbiol.* **2005**, *13*, 119–127.
474 23. Irazoqui, J. E.; Urbach, J. M.; Ausubel, F. M. Evolution of host innate defence:
475 insights from Caenorhabditis elegans and primitive invertebrates. *Nature reviews.*
476 *Immunology* **2010**, *10*, 47–58.
477 24. Kim, D. H.; Feinbaum, R.; Alloing, G.; Emerson, F. E.; Garsin, D. A.; Inoue, H.;
478 Tanaka-Hino, M.; Hisamoto, N.; Matsumoto, K.; Tan, M.-W.; Ausubel, F. M. A
479 conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity.
480 *Science* **2002**, *297*, 623–626.
481 25. Ermolaeva, M. A.; Schumacher, B. Insights from the worm: The C. elegans model
482 for innate immunity. *Seminars in Immunology* **2014**, *26*, 303–309.
483 26. Youngman, M. J.; Rogers, Z. N.; Kim, D. H. A Decline in p38 MAPK Signaling
484 Underlies Immunosenescence in Caenorhabditis elegans. *PLOS Genet* **2011**, *7*,
485 e1002082.
486 27. Ortega-Riveros, M.; De-la-Pinta, I.; Marcos-Arias, C.; Ezpeleta, G.; Quindós, G.;
487 Eraso, E. Usefulness of the Non-conventional Caenorhabditis elegans Model to
488 Assess Candida Virulence. *Mycopathologia* **2017**, *182*, 785–795.
489 28. Pukkila-Worley, R.; Ausubel, F. M.; Mylonakis, E. Candida albicans Infection of
490 Caenorhabditis elegans Induces Antifungal Immune Defenses. *PLoS Pathog* **2011**,
491 *7*, e1002074–13.
492 29. Moy, T. I.; Ball, A. R.; Anklesaria, Z.; Casadei, G.; Lewis, K.; Ausubel, F. M.
493 Identification of novel antimicrobials using a live-animal infection model. *Proc Natl*
494 *Acad Sci USA* **2006**, *103*, 10414–10419.

495 30. Peleg, A. Y.; Tampakakis, E.; Fuchs, B. B.; Eliopoulos, G. M.; Moellering, R. C.;
496 Mylonakis, E. Prokaryote-eukaryote interactions identified by using Caenorhabditis
497 elegans. *Proc Natl Acad Sci U S A* **2008**, *105*, 14585–14590.
498 31. Muschiol, D.; Schroeder, F.; Traunspurger, W. Life cycle and population growth rate
499 of Caenorhabditis elegans studied by a new method. *BMC Ecol.* **2009**, *9*, 14.
500 32. Corsi, A. K.; Wightman, B.; Chalfie, M. A Transparent Window into Biology: A Primer
501 on Caenorhabditis elegans. *Genetics* **2015**, *200*, 387–407.
502 33. Abbate, J. L.; Kada, S.; Lion, S. Beyond Mortality: Sterility As a Neglected
503 Component of Parasite Virulence. *PLoS Pathog* **2015**, *11*, e1005229.
504 34. Read, A. F. The evolution of virulence. *Trends Microbiol.* **1994**, *2*, 73–76.
505 35. Lipsitch, M.; Siller, S.; Nowak, M. A. The evolution of virulence in pathogens with
506 vertical and horizontal transmission. *Evolution* **1996**, *50*, 1729–1741.
507 36. Cressler, C. E.; McLEOD, D. V.; Rozins, C.; van den Hoogen, J.; Day, T. The
508 adaptive evolution of virulence: a review of theoretical predictions and empirical
509 tests. *Parasitology* **2016**, *143*, 915–930.
510 37. Gillum, A. M.; Tsay, E. Y.; Kirsch, D. R. Isolation of the *Candida albicans* gene for
511 orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and
512 *E. coli pyrF* mutations. *Mol Gen Genet* **1984**, *198*, 179–182.
513 38. Morschhäuser, J.; Ruhnke, M.; Michel, S.; Hacker, J. Identification of CARE-2-
514 negative Candida albicans isolates as Candida dubliniensis. *Mycoses* **1999**, *42*, 29–
515 32.
516 39. Jain, C.; Pastor, K.; Gonzalez, A. Y.; Lorenz, M. C.; Rao, R. P. The role of Candida
517 albicans AP-1 protein against host derived ROS in in vivo models of infection.
518 *Virulence* **2013**, *4*, 67–76.
519 40. Yapar, N. Epidemiology and risk factors for invasive candidiasis. *Ther Clin Risk*
520 *Manag* **2014**, *10*, 95–105.
521 41. Krcméry, V.; Barnes, A. J. Non-albicans Candida spp. causing fungaemia:
522 pathogenicity and antifungal resistance. *J. Hosp. Infect.* **2002**, *50*, 243–260.
523 42. Meisel, J. D.; Kim, D. H. Behavioral avoidance of pathogenic bacteria by
524 Caenorhabditis elegans. *Trends Immunol.* **2014**, *35*, 465–470.

525 43. Jain, C.; Yun, M.; Politz, S. M.; Rao, R. P. A pathogenesis assay using
526 Saccharomyces cerevisiae and Caenorhabditis elegans reveals novel roles for yeast
527 AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis. *Eukaryotic Cell*
528 **2009**, *8*, 1218–1227.
529 44. Gravato-Nobre, M. J.; Nicholas, H. R.; Nijland, R.; O'Rourke, D.; Whittington, D. E.;
530 Yook, K. J.; Hodgkin, J. Multiple genes affect sensitivity of Caenorhabditis elegans to
531 the bacterial pathogen Microbacterium nematophilum. *Genetics* **2005**, *171*, 1033–
532 1045.
533 45. Garsin, D. A.; Villanueva, J. M.; Begun, J.; Kim, D. H.; Sifri, C. D.; Calderwood, S.
534 B.; Ruvkun, G.; Ausubel, F. M. Long-lived C. elegans daf-2 mutants are resistant to
535 bacterial pathogens. *Science* **2003**, *300*, 1921–1921.
536 46. Pfaller, M. A.; Diekema, D. J.; Gibbs, D. L.; Newell, V. A.; Ellis, D.; Tullio, V.;
537 Rodloff, A.; Fu, W.; Ling, T. A.; Global Antifungal Surveillance Group Results from
538 the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year
539 analysis of susceptibilities of Candida Species to fluconazole and voriconazole as
540 determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* **2010**, *48*, 1366–
541 1377.
542 47. Vilela, M. M. S.; Kamei, K.; Sano, A.; Tanaka, R.; Uno, J.; Takahashi, I.; Ito, J.;
543 Yarita, K.; Miyaji, M. Pathogenicity and virulence of Candida dubliniensis:
544 comparison with C. albicans. *Med Mycol* **2002**, *40*, 249–257.
545 48. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J.
546 Candida glabrata, Candida parapsilosis and Candida tropicalis: biology,
547 epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* **2012**,
548 *36*, 288–305.
549
550

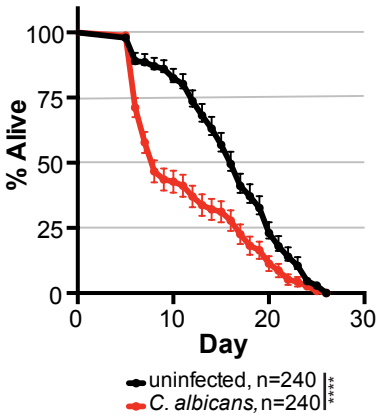

Figure 1: Reduced nematode survival associated with *C. albicans* infection. Survival curves for *C. elegans* populations that are either uninfected (exposed to just an *E. coli* food source, black) or when infected with *C. albicans* SC5314 (red). Mean data values are plotted with SEM error bars, number of worms analyzed (n) for each treatment is indicated in the legend. **** indicates $p < 0.0001$, Log-rank (Mantel-Cox) test.

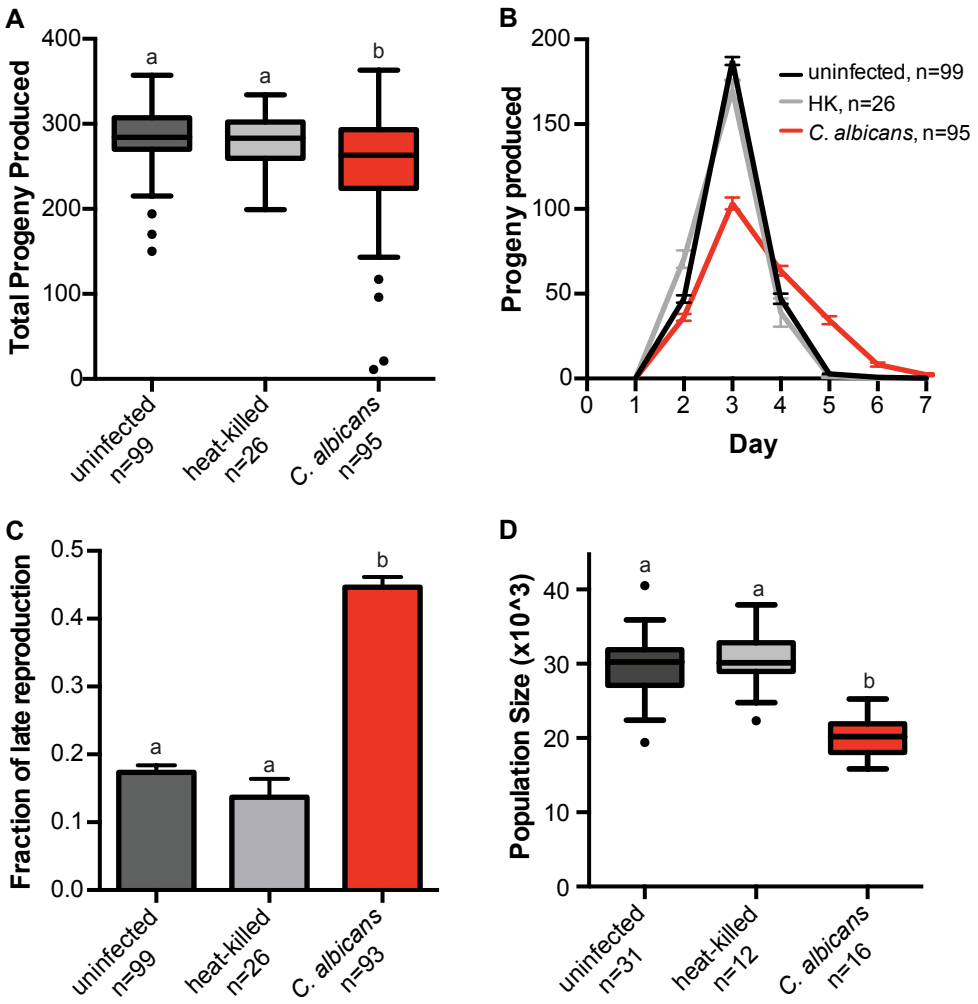

Figure 2. Live *C. albicans* reduces nematode fecundity. A) Box and whiskers plot of brood sizes for *C. elegans* (N2) exposed to *E. coli* food source alone (uninfected, black), heat-killed *C. albicans* (grey), or live *C. albicans* (red). Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. **B)** Number of viable *C. elegans* progeny produced per day in uninfected, heat-killed, and live *C. albicans* treatments. Data represent the mean and SEM. **C)** The fraction of *C. elegans* progeny produced on Day4 or later during the 'Late Reproductive' window. Data represent the mean and SEM. **D)** Box and whiskers plot of average population size (representing the number of F1 and F2 progeny) produced from a single founder *C. elegans*. Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. The number (n) of experimental samples analyzed is indicated for each treatment. Treatments that share letters are not significantly different, whereas treatments with differing letters are stastically significant, post-hoc Dunn's multiple comparison test.

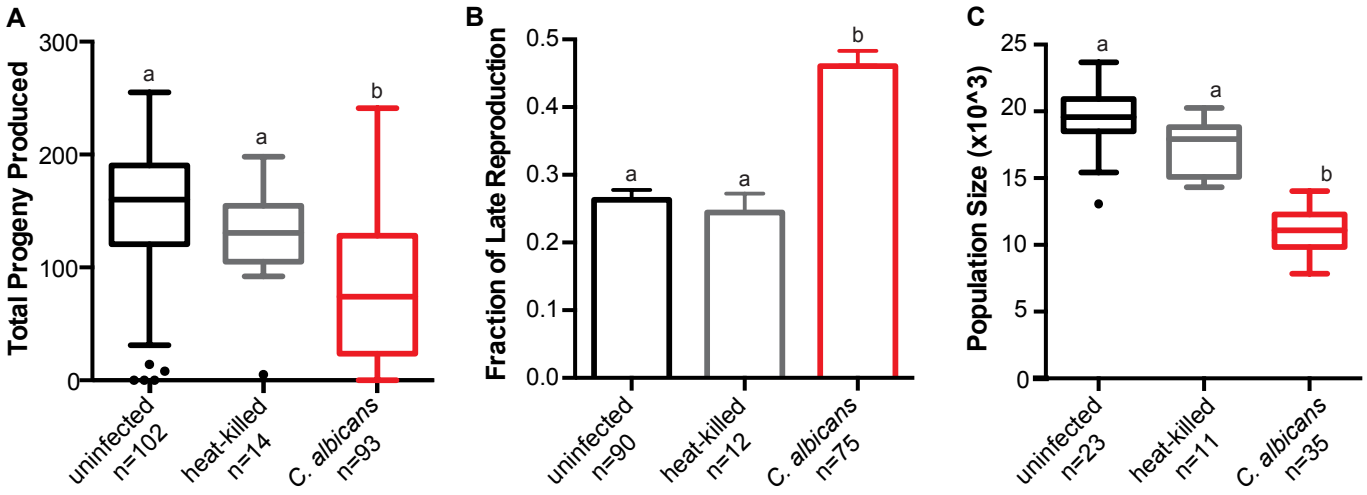

Figure 3: *C. albicans* severely impacts host fitness in immunocompromised nematodes. **A)** Box and whiskers plot of brood sizes for *C. elegans* (N2) exposure to *E. coli* food source alone (uninfected, black), heat-killed *C. albicans* (grey), or live *C. albicans* (red). Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. **B)** The fraction of *C. elegans* progeny produced on Day 4 or later during the 'Late Reproductive' window. Data represent the mean and SEM. **C)** Box and whiskers plot of average population size (representing the number of F1 and F2 progeny) produced from a single founder *C. elegans*. Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data and circles indicate outliers. The number (n) of experimental samples analyzed is indicated for each treatment. Stastistical significance is indicated when treatments differ. Treatments that share letters are not significantly different, whereas treatments with differing letters are stastically significant, post-hoc Dunn's multiple comparison tests.

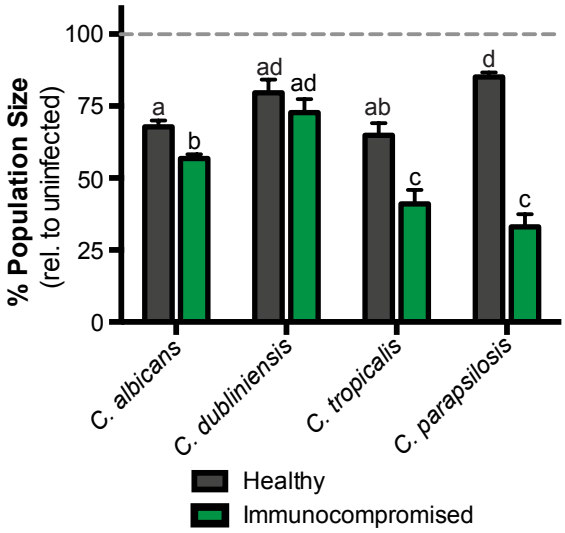

Figure 4. Virulence of *Candida* species depends on host immune function.
Reductions in relative population size for healthy (N2, black bars) and immunocompromised (*sek-1*, green bars) hosts exposed to *C. albicans*, *C. dublineinsis*, *C. tropicalis*, and *C. parapsilosis*. Dashed line indicates the relative population size of uninfected hosts. Interactions between *Candida* species and host background was determined by two-way ANOVA ($p < 0.001$). Treatments that share letters are not significantly different, whereas treatments with differing letters are stastically significant, post-hoc Tukey's multiple comparisons test. The number (n) of experimental samples analyzed is indicated for each treatment in Table S1.