The little caterpillar that could – Tobacco Hornworm (*Manduca sexta*) caterpillars as a novel host model for the study of fungal virulence and drug efficacy

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Abstract

Pathogenic yeast species can cause life-threatening infections in humans. The two leading yeast pathogens, *Candida albicans* and *Cryptococcus neoformans*, cause systemic infections in >1.4 million patients world-wide with mortality rates approaching 75%. It is thus imperative to study fungal virulence mechanisms, stress response pathways, and the efficacy of antifungal drugs. This is commonly done using mammalian models. To address ethical and practical concerns, invertebrate models, such as wax moth larvae, nematodes, or flies, have been introduced over the last two decades. To address short-comings in existing invertebrate host models, we developed fifth instar caterpillars of the Tobacco Hornworm moth *Manduca sexta* as a novel host model for the study of fungal virulence and drug efficacy. These caterpillars can be raised at standardised conditions, maintained at 37˚C, can be injected with defined amounts of yeast cells, and are susceptible to the most threatening yeast pathogens, including *C. albicans*, *C. neoformans*, *C. auris*, and *C. glabrata*. Infected caterpillars can be rescued by treatment with commonly deployed antifungal drugs and importantly, fungal burden can be assessed daily throughout the course of infection in a single caterpillar's faeces and hemolymph. Notably, these animals are large enough so that weight provides a reliable and reproducible measure of fungal virulence. This model combines a suite of parameters that recommend it for the study of fungal virulence.

Introduction

Fungal infections pose a serious threat to human health and well-being world-wide. Each year, as many, if not more patients, die of fungal infections than of malaria or tuberculosis[1]. The leading yeast pathogens, *Candida albicans* and *Cryptococcus neoformans*, together account for >1,400,000 life-threatening infections world-wide with mortality rates approaching 75%[1]. Candidemia, most commonly caused by *C. albicans*, is the fourth most common cause of nosocomial blood stream infections, only surpassed by infections with Staphylococci and *Enterococcus* spp. Disturbingly, candidemia incidence rates are on the rise. Within less than ten years, incidence rates increased by 36%[2]. Although cryptococcosis incidence rates are on the decline in North America, this AIDS-defining illness is responsible for 15% of all AIDS-related deaths world-wide[2,3]. This already dire situation is further confounded by the emergence of drug resistant yeast species. Patients at risk of developing invasive candidemia are often prophylactically treated with fluconazole, while the echinocandins are considered a first line defence strategy[4]. Yet, *C. glabrata*, the most

common non- *albicans Candida* species associated with nosocomial blood stream infections[5], is intrinsically less susceptible to azole drugs and acquires resistance to echinocandins rapidly[6]. The rapid global spread of multi-drug resistant *C. auris* has further exacerbated the threat posed by fungi. *C. auris* was first reported in 2009 in Japan[7]. In 2015, *C. auris* arrived in Europe causing an outbreak involving 72 patients in a cardio-thoracic hospital in London[8]. *C. auris* outbreaks have been reported from South Korea, India, Spain, Columbia, Switzerland, Germany, Israel, Kuweit, and Oman[9]. Most concerningly, up to 25% of *C. auris* isolates are multi-drug resistant, with some strains being resistant to three of the four drug classes available for the treatment of systemic candidemia. In addition to the unacceptably high burden on human health, fungal infections substantially increase health care costs. Treatment requires extended hospitalisation, resulting in additional costs of up to $45,000 in adult patients or up to $119,000 in paediatric patients in the case of candidemia[10].

It is thus imperative to investigate fungal virulence and host response mechanisms. This is traditionally done in mammalian models. The most frequently employed models include the mouse tail vain infection model for systemic candidemia, the mouse gastrointestinal infection model of candidemia, the mouse *Candida* vaginitis model[11], the mouse inhalation model of cryptococcosis[12], the rabbit chronic cryptococcal meningitis model[13], and the rabbit *Candida* keratitis model[14]. While mammalian models combine a number of features that make them particularly amenable for the study of fungal diseases, such as susceptibility, availability of knock-out mutants, and comparable histology to human disease, using mammals is ethically controversial, economically challenging, and requires extensive board certifications and documentations. Furthermore, specific applications, such as the screening of large-scale fungal mutant libraries are not sustainable in mammalian models.

In an effort to reduce the usage of mammals as model hosts, alternative invertebrate models have been developed and used in fungal virulence research over the past two decades. The most commonly employed invertebrate species include the nematode *Caenorhabditis elegans*, the fly *Drosophila melanogaster*, and larvae of the Greater Wax moth *Galleria mellonella*. All three species can be easily maintained in the laboratory at a much lower cost than mice or rabbits and have been successfully used for the study of diverse yeast pathogens, such as *C. neoformans*[15,16], *C. albicans*[16-18], *C. parapsilosis*[18-20], *C. glabrata*[19,21]. Of note, invertebrate models differ in their applicability and the best suitable model should be

94 carefully selected[22]. Unlike mammalian host models, these invertebrates do not have adaptive
95 immunity but all have components of the innate immune system[23,24], some of which are
96 conserved with mammals. This includes the Toll-like receptors found in the fly[25] and the
97 homolog of the MKK3/6 kinase in the nematode[26]. Ironically, it is the Toll-like receptors that
98 protect flies from infections with *C. neoformans*[27], *C. albicans*[28], and *C. glabrata*[29] and the
99 MKK3/6 homolog SEK-1 protects the nematode from bacterial invaders[26]. Thus, to increase
100 susceptibility of flies and nematodes to fungal pathogens, Toll and *sek-1*[30] mutants need to be
101 used. A key limitation for the study of human pathogens, is the inability of the nematode and
102 the fly to survive human body temperature. Only *Galleria* can withstand 37˚C[16]. The *Galleria*
103 genome has been announced very recently[31], yet a detailed analysis, including annotations, is
104 still missing. Due to their long-standing history as eukaryotic models, well-curated genomes
105 and genome databases exist for the nematode and the fly. Yet, neither organism allows for the
106 delivery of an exact inoculum of fungal cells, only *Galleria* can be directly injected with a
107 defined cell number. Until recently, *Galleria* larvae for research had to be purchased from
108 fishing shops. Now, UK-based TruLarv is selling research grade larvae while extensive stock
109 collections exist for the fly and nematode.

110 An insect model with a long history in research, the Tobacco Hornworm *Manduca*
111 *sexta*, has yielded important insights into flight mechanisms, nicotine resistance, hormonal
112 regulation of development, metamorphosis, antimicrobial defences, and bacterial
113 pathogenesis. *M. sexta* laboratory stocks have been derived from animals collected in North
114 Carolina, USA[32] and been maintained in laboratories on both sides of the Atlantic for several
115 decades. In the wild, *M. sexta* is most commonly encountered in the southern United States,
116 where it feeds on solanaceous plants and is thus considered a plant pest. *M. sexta*'s research
117 portfolio includes innate immunity[33], a genome sequence that has been complemented with
118 tissue-specific transriptomic analyses[34], numerous successful applications of RNAi[35-39], and
119 protocols for the efficient extraction of hemocytes for down-stream analyses[40]. But despite its
120 versatility and prominent role in bacterial pathogenesis research, *M. sexta* has yet to be
121 explored for its suitability as a host model for fungal infections.

122 Here, we aimed to establish *M. sexta* as a novel model host for the study of fungal
123 virulence. Inbred animals from the University of Bath's research colony, that has been
124 established in the 1980s, were tested for their ability to live at 37˚C, their susceptibility to
125 different yeast species, and the reproducibility of *C. albicans* mutant phenotypes obtained in

126 mice virulence studies. Indeed, the caterpillars grow at 37˚C while maintaining susceptibility,
127 specific *C. albicans* mutants are just as attenuated in their virulence in *M. sexta* as they are in
128 mice, and notably, *M. sexta* are susceptible to the leading yeast pathogens *C. albicans*, *C.*
129 *neoformans*, as well as the emerging *C. auris*. To expand *M. sexta's* applicability as a host
130 model, we developed an infection protocol that permits screening of fungal burden
131 throughout the course of infection in a single animal and uses weight as a proxy measure for
132 virulence in addition to survival. *M. sexta* can furthermore be used to test efficacy of common
133 antifungal drugs. Our results define *M. sexta* characteristics that recommend the caterpillars
134 as a non-mammalian host model for the study of fungal virulence.

135 **Materials and Methods**

136 **Origin of the Bath colony of *Manduca sexta***

137 The colony has been in continuous culture since 1978 without the addition of animals
138 from elsewhere. Bath's genetic stock was derived from animals from the Truman-Riddiford
139 laboratories at the University of Washington in Seattle, USA. Their animals date back to the
140 ones originally collected in North Carolina in 1976[32].

141 .

142 **Caterpillar and yeast culture conditions**

143 *M. sexta* caterpillars were reared to fifth instar under standardised conditions. They
144 were maintained in 125 ml disposable cups (Sarstedt Ltd., Cat. No. 75.1335), on a wheat
145 germ-based diet (Appendix 1), at a constant temperature of 25˚C with 50% humidity, and 12
146 hours of light and dark cycles. Three days prior to infections with fungi, animals were shifted
147 to a formaldehyde-free diet as the compound is toxic to non-methylotrophic yeast.

148 For infection assays, yeasts were grown overnight in 50 ml YPD (1% yeast extract,
149 2% peptone, 2% dextrose) and cells harvested by centrifugation for 3 minutes at 3,000 rpm.
150 The cell pellet was washed twice with 1x phosphate buffered saline (PBS) and suspended in 5
151 ml 1x PBS. Cells were counted and numbers adjusted as indicated. *C. albicans* YSD85
152 (Table 1) cells were heat-inactivated by incubation at 65˚C for 20 minutes. For long-term
153 storage, yeast isolates were maintained at -80˚C in 25% glycerol.

154 **Yeast infections and measurements of fungal burden and drug efficacy**

155 100 µl of washed and number-adjusted yeast suspension were injected into each
156 caterpillar with a 30G1/2" needle (BD Microlance) and a 1 ml NORM-JECT syringe.
157 Animals were injected through their distal left proleg. Following injection, each animal's
158 weight was recorded. Animals were scored for survival and weight once daily for three to
159 four days post infection. During the course of the experiment, animals were kept on a 12 hour
160 light and dark cycle at the temperature indicated and on their regular diet.

161 To measure fungal burden in caterpillar faeces and hemolymph, six animals were
162 injected with either 1x PBS or 10^6 cells of the wild type YSD89 or the *hog1* mutant strain
163 YSD883 and kept at 37˚C. On day 1, two animals were selected from each group. These
164 animals were weighted and their hemolymph and faeces collected daily throughout the course
165 of infection. To collect hemolymph, animals were first kept on ice for 15 minutes. The 'horn'
166 was then surface sterilised with 70% ethanol and its top 1-2 mm clipped with a pair of micro
167 scissors. Hemolymph was collected in a pre-chilled 1.5 ml Eppendorf tube and cooled
168 immediately to reduce polymerisation and melanisation. One faecal pellet was collected daily
169 with sterile forceps, weighted and suspended in 500 µl 1x PBS. Prior to diluting, the mixture
170 was thoroughly vortexed for 10 seconds, and centrifuged for 5 seconds using a table top
171 centrifuge to separate faecal matter. To quantify fungal burden, hemolymph and faecal
172 samples were plated either directly onto YPD-agar with Kanamycin 50 µg/ml or in ten-fold
173 serial dilutions. Agar plates were incubated at 30˚C for 48 hours and colonies counted.

174 To assess the efficacy of commonly used antifungal drugs, animals were infected with
175 10^7 cells of YSD85 or PBS and treated with increasing doses of fluconazole and caspofungin
176 (Sigma Aldrich, Inc.) as indicated. Drugs were injected with an ethanol-sterilized Hamilton
177 syringe in a total volume of 10 µl per animal, 30 minutes post-infection. Caterpillars were
178 weighted and scored for survival on the day of injection and the following three days.

179 **Statistical analyses**

180 Survival plots were made using the **survminer** R package (https://CRAN.R-
181 project.org/package=survminer), and differences were evaluated using the Kaplan-Meier
182 method. Weight and fungal burden were plotted using ggplot2[41] and weight differences were
183 evaluated using linear models with day post-inoculation and the interaction between
184 treatment and dpi as fixed effects and individual as a random effect using nlme

185 (https://CRAN.R-project.org/package=nlme). All analyses were done using RStudio version
186 1.1.442.

187 **Results**

188 We first aimed to determine if *M. sexta* fifth instar caterpillars, reared and maintained
189 at standard conditions (Fig. 1a), are susceptible to *Candida albicans*. To do so, groups of ten
190 animals were infected with increasing doses of the widely used *C. albicans* laboratory strains
191 SC5314 and SN95[42]. Animals were scored daily for survival for three consecutive days while
192 being maintained at 25˚C. Dead animals differ from live ones in that their bodies go limp and
193 turn grey-green in colour, which is in stark contrast to the vivid turquoise of live animals
194 (Fig. 1b). Indeed, caterpillars that were infected with *C. albicans* succumbed to the yeast in a
195 dose-dependent manner. Both *C. albicans* strains killed *M. sexta* caterpillars efficiently at
196 inocula of 10^6 or 10^7 cells per animal (Fig. 1c). To determine if survival measures in
197 caterpillars are comparable to those obtained in the current gold standard, the murine model
198 of systemic candidemia, we tested *C. albicans* mutants with published phenotypes of either
199 attenuated virulence, such as the *hog1*Δ/Δ[43] and *ahr1*Δ/Δ[44] mutants, or wild-type levels of
200 virulence, such as *cka2*Δ/Δ[45]. Cross-species virulence levels are comparable for Hog1, which
201 is as essential for virulence in caterpillars as it is in mice. Cka2 is not required to establish
202 systemic infections in mammals but is in caterpillars. Ahr1, while required for virulence in
203 mammals, appears to be dispensable for virulence in caterpillars (Fig. 1d).

204 Given the importance of temperature for fungal virulence, we aimed to determine if
205 *M. sexta* retained their susceptibility to *C. albicans* at human body temperature of 37˚C.
206 Temperature itself does not affect caterpillar survival or development (Fig. S1) but animals
207 are ten times more susceptible to infections with *C. albicans* at 37˚C than they are at 25˚C
208 (Fig. 2a). At 37˚C, 10^6 *C. albicans* cells per animal lead to 100% mortality on day 4, while
209 10^7 cells are required for the same outcome at 25˚C (Fig. 1b). To exclude the possibility that
210 mortality is due to starvation rather than the outcome of a host-pathogen interaction, we
211 infected caterpillars with live and heat-killed *C. albicans* wild-type cells at 37˚. Only live
212 cells, but not heat-killed *Candida* cells, kill caterpillars suggesting that killing is not due to
213 nutritional limitations (Fig. S2). Demonstrating susceptibility of *M. sexta* caterpillars to
214 *Candida albicans* supports their suitability as an alternative host model for the study of
215 fungal virulence but also highlights the need for additional measures of fungal virulence. To
216 add granularity to fungal virulence data collected from *M. sexta*, we complemented measures

217 of survival with quantifications of weight and fungal burden throughout the course of
218 infection. To collect weight data, caterpillars were weighted prior to infection and then daily
219 throughout the course of infection. Weight gain in animals infected with a low dose of 10^4
220 cells did not significantly differ from those injected with 1x PBS but caterpillars infected
221 with10^5 cells per animal exhibited significant weight loss. Too few animals survived
222 infection with 10^6 cells to allow for a meaningful comparison (Fig. 2b). After establishing
223 susceptibility of *M. sexta* to *C. albicans* at 37˚C, we aimed to validate the attenuated
224 virulence phenotype of the *hog1*Δ/Δ mutant strain. To test this, caterpillars were infected with
225 10^6 cells per animal with the wild type strain, the *hog1*Δ/Δ mutant, and the complemented
226 strain *hog1/hog1::HOG1* and compared to the control group injected with 1x PBS. The
227 *hog1*Δ/Δ mutant is less virulent than the wild type (Fig. 2c). Animals infected with the
228 *hog1*Δ/Δ mutant strain JC 50 gained significantly more weight than those infected with the
229 wild type RM1000, while infection with the complemented strain JC52 leads to a comparable
230 lack in weight gain (Fig. 2d).

231 To further expand the applicability of *M. sexta* caterpillars as a host model for fungal
232 infections, fungal burden in the hemolymph and faeces was quantified daily throughout the
233 course of infection. Since neither the collection of hemolymph nor that of faeces necessitates
234 killing of the animal, data could be collected daily throughout the infection for the same
235 caterpillar. Animals infected with the wild type and the *hog1*Δ/Δ mutant strain YSD883 were
236 compared to control animals injected with 1x PBS only. Analysing fungal burden by counting
237 colony-forming units in faeces and hemolymph in two animals per group, revealed an
238 increase in CFU counts in the animals infected with the wild type but not the *hog1*Δ/Δ mutant
239 or the PBS-control group (Figs. 3a, b). While no yeasts were detected in either group one day
240 post infection (p.i), CFUs in faeces and hemolymph are detectable two days p.i. in animals
241 injected with the wild type. Three days p.i., the CFU count in the wild-type group increase
242 while CFUs are detected for the first time in the *hog1*Δ/Δ group. In keeping with the increase
243 in fungal burden, weight gain in the wild-type infected group was reduced, while *hog1*Δ/Δ
244 infected animals gained weight at a similar rate to the control animals (Fig. 3c). Thus,
245 measurements of fungal burden, that can be obtained from the same animal throughout the
246 course of infection, provide a valuable parameter for the study of fungal virulence.

247 The currently available armamentarium of antifungal drugs is limited and often
248 lacking in efficacy. Studying drug efficacy and drug mode of action in the host are thus

249 pertinent to the much needed development of novel antifungal drugs and further
250 characterisation of existing compounds. To establish suitability of *M. sexta* as a model for
251 drug efficacy testing, we recorded survival and weight of animals infected with the *C.*
252 *albicans* wild-type strain SC5314 and treated with increasing doses of two common
253 antifungals, fluconazole and caspofungin (Fig.4). Treatment with fluconazole or caspofungin
254 resulted in overall improved survival and weight gain. Pair-wise comparisons between
255 different drug concentrations with the untreated control animals yielded no statistical
256 significance for the fluconazole-treated group of caterpillars but those being treated with 2
257 mg/kg and 4 mg/kg of caspofungin survived significantly better than those without treatment
258 or those that only received 1 mg/kg of caspofungin (Table 2).

259 While *C. albicans* undoubtedly is a leading fungal pathogen of humans, clinical
260 manifestations of fungal infections are not limited to *C. albicans*. To broaden *M. sexta*'s
261 applicability, we sought to determine the caterpillars' susceptibility to other fungal
262 pathogens. To this end, animals were infected with *C. neoformans*, *C. auris*, and *C. glabrata*
263 in addition to *C. albicans*. Type strains of *Saccharomyces cerevisiae*, the baker's or brewer's
264 yeast and *Metschnikowia pulcherrima*, a yeast inhabiting fruits and flowers[46] served as
265 reference points for attenuated virulence infections. Animals were infected with increasing
266 doses of yeast cells starting at 10^5 cells per animal and up to 10^9 cells per animal. Groups of
267 ten animals per yeast cell dose and species were then screened for survival and weight daily
268 (Figs. 5 and 6). Notably, only infections with pathogenic yeast species caused reduced
269 survival of caterpillars (Fig. 5). Infections with *S. cerevisiae* or *M. pulcherrima* did not affect
270 survival of caterpillars. Comparing survival amongst the pathogenic yeast species revealed *C.*
271 *albicans* to be the most severe. 10^7 *C. albicans* cells per animal result in 100% killing within
272 in 24 hours. 10^9 *C. auris* cells were required to kill all animals within four days and the same
273 dose of *C. glabrata* resulted in 75% killing. 10^8 *C. neoformans* cells were required to achieve
274 50% killing within four days, comparable to survival of animals infected with *C. glabrata*. It
275 should be noted that due to high viscosity of the cell suspension, we could not test higher
276 concentrations than the ones stated. In addition to assessing survival, each surviving
277 caterpillar's weight was recorded daily. Interestingly, while infections with *S. cerevisiae* or
278 *M. pulcherrima* did not kill caterpillars, infected animals displayed reduced weight gain (Fig.
279 6). Infections with *C. albicans* resulted in severely reduced weight gain, even at the lowest
280 yeast dose tested. Animals infected with *C. auris* and *C. glabrata* responded in a dose-
281 dependent manner, the higher the yeast dose, the more dramatic the weight loss. Notably,

282 animals infected with the lowest dose of *C. neoformans* grew better than PBS-injected control
283 animals. Increasing the yeast dosage, however, resulted in significantly reduced weight gain.
284 This bi-phasic pattern, resembling hormesis[47] in which a low dose of an environmental agent
285 is beneficial, while a high dose is toxic, could be due to *C. neoformans*' immunogenic
286 capsule[48]. Caterpillars displaying variable degrees of susceptibility to different yeast species,
287 with weight providing an additional measure of host damage, suggests that this host model is
288 broadly applicable for the study of fungal virulence.

289 **Discussion**

290 *M. sexta* caterpillars are naturally susceptible to the leading human yeast pathogens.
291 Unlike other host models, they permit assessment of fungal burden daily throughout the
292 course of infection in a single animal by either collecting faeces or hemolymph. *M. sexta* can
293 be maintained at 37˚C, is large enough to be injected with a specified yeast inoculum and for
294 weight to be a reliable measure of virulence. *C. albicans* mutant virulence phenotypes found
295 in mice can be replicated and yeast inocula required to elicit a response in caterpillars are
296 comparable to those used in the murine model. Additionally, the caterpillars permit study of
297 antifungal drug efficacy. These parameters commend this invertebrate as a novel host model
298 for the study of fungal virulence.

299 This new model system allows for fungal burden to be monitored throughout the
300 course of infection in a single animal via CFU count. This is unlike any other experimental
301 system, where fungal burden is either an endpoint measure in the mouse kidney, in
302 homogenised was moth larvae[49],nematodes[50], flies[51], or requires genetically modified
303 fluorescent yeast strains for microscopic imaging and analyses[52]. While we did detect very
304 low CFU counts in the PBS control 3 days post infection, we consider this spurious finding
305 due to cross contamination as preliminary experiments of plating contents of hemolymph and
306 faeces of naïve animals did not detect any yeast growth (data not shown). As a consequence,
307 we amended to protocol to include changing gloves when handling animals of different
308 treatment groups.

309 When reviewing the weight data collected as part of our study, we noticed two
310 interesting aspects. First, while *S. cerevisiae* and *M. pulcherrima* do not affect caterpillar
311 survival, infections with either species led to reduced weight gain. The dichotomy between
312 survival and weight observed here, further emphasizes that fungal virulence comprises more

313 than a measure of survival. It appears that *M. sexta* would allow to discrimination between
314 disease (weight) and death (survival) quantitatively adding further granularity to measuring
315 fungal virulence. Secondly, infections with *C. neoformans* resulted in increased caterpillar
316 weight gain at a low yeast dose but reduced weight gain at higher doses. This pattern
317 resembles the concept of hormesis often deployed by toxicologists to describe the response to
318 toxins, where exposure to a low dose is beneficial while a higher dose results toxicity due to
319 overcompensation in response to disruption of homeostasis[53]. While tissue-specific hormetic
320 responses have been described in flies, where a virus-acquired cytokine relays ageing[54], and
321 examples of abiotic factors or signalling molecules affecting peas and aphid infestation[55],
322 immunity in plants[56,57], life-span in malaria-transmitting *Anopheles*[58], and larval development
323 in Black Cutworm[59], this example here could be the first involving a eukaryotic host-
324 pathogen relationship. The underlying factor remains to be elucidated but *C. neoformans*'
325 highly immunogenic capsule seems to be an excellent candidate[48].

326 Heat-killed *C. albicans* cells appear to be entirely non-pathogenic in *M. sexta*
327 caterpillars as neither survival nor weight are affected by innoculation with heat-killed yeast
328 cells. The lack of mortality in response to innoculation with heat-killed yeast cells indicates
329 that yeast viability and proliferation are required for pathogenesis and excludes the possibility
330 of death due to an allergic reaction in response to a large number of fungal cells. This appears
331 to differ from the responses of other host models to fungal pathogens. While susceptibility
332 was reduced, but still measurable, in *G. mellonella*[60] and the two-spotted cricket[61], heat-killed
333 *C. albicans* cells elicited 100% mortality in a sepsis-like murine model[62]. In mice, serum
334 levels of β-(1,3)-glucan levels were elevated in animals injected with heat-killed yeast cells
335 when compared to those infected with live cells. Indeed, heat inactivation leads to increased
336 exposure of β-(1,3)-glucan on the *C. albicans* cell surface[63] and β-(1,3)-glucan activates the
337 innate immune response in invertebrates and mammals[64]. Given the complexity of receptors
338 involved in recognition of fungal invaders[65] and the lack of a response in *M. sexta* and other
339 invertebrates indicates that they may not host all or identical receptors as mammals.

340 We showed that *Manduca sexta* caterpillars expand the repertoire of invertebrate
341 models for the study of fungal disease. It combines the unique advantage of allowing
342 measurement of fungal burden with standard quantifications of survival and weight.
343 Although, *M. sexta* genomic and transcriptomic analyses are currently still in their infancy,
344 we would expect that the Tobacco Hornworm's long history of being an invaluable model for

diverse facets of biology will lead to reliable tools in combination with genetic tractability and protocols establishing the yeasts' fate inside the caterpillar.

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Disclosure of interest

The authors report no interest of conflict.

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577 **Appendices**

578 **Appendix 1 – Food preparation**

579

Premix		Diet cake	
Amount	Ingredient	Amount	Ingredient
2,700 g	Wheatgerm	336 g	Premix
1,260 g	Casein	1,770 ml	Distilled water
1,080 g	Sucrose	22.5 g	Agar
540 g	Dried active yeast	4 ml	Corn oil
360 g	Wesson's Salt	4 ml	Linseed oil
36 g	Choline chloride	8 ml	4% Formaldehyde
72 g	Cholesterol	0.2 g	Chlorotetracycline
36 g	Methyl paraben	0.2 g	Vanderssant vitamins
54 g	Sorbic acid	8 g	Ascorbic acid

580 For the premix, deactivate the yeast by microwaving for 5 minutes on low power
581 before mixing all components thoroughly. Store in a cool, dry place.

582 For the diet cake, heat up 650 ml of water on a hot plate while melting the agar in 1 l
583 of water by microwaving. Combine the agar with the pre-warmed water, 336 g of premix,
584 formaldehyde and oils and mix thoroughly using a stand mixer. Dissolve vitamins, antibiotic,
585 and ascorbic acid in the remaining 30 ml of water and add to mixing bowl once the content
586 has cooled below 50˚C to prevent inactivation of vitamins and antibiotic. Line a large ice
587 cube tray with sterile aluminium foil (sterilise by spraying with 70% ethanol) and pour
588 mixture into tray. Let the diet mix set for about 1.5 hours, wrap tightly in aluminium foil and
589 store at 4˚C. Keeps for 3 weeks.

590

591 **Tables**

592 **Table 1:** Yeast strains used in this study.

Strain ID	Strain Name	Species	Source
YSD89	SN95	*C. albicans*	
YSD85	SC5314	*C. albicans*	
YSD883	hog1Δ/Δ	*C. albicans*	This study
YSD87	BWP17	*C. albicans*	[66]
YSD190	VIC84 (cka2Δ/Δ)	*C. albicans*	[67]
YSD192	VIC93 (cka2/cka2::CKA2)	*C. albicans*	[67]
YSD302	CAS12 (ahr1Δ/Δ)	*C. albicans*	[44]
YSD303	CAS13 (ahr1/ahr1::AHR1)	*C. albicans*	[44]
YSD304	RM1000	*C. albicans*	[68]
YSD305	JC52 (hog1/hog1::HOG1)	*C. albicans*	[68]
YSD306	JC50 (hog1Δ/Δ)	*C. albicans*	[68]
YSD790	YPS143	*S. cerevisiae*	[69]
YSD1448	NCYC2580	*M. pulcherrima*	[70]
YSD465	2001	*C. glabrata*	[71]
YSD1454	TA004-14	*C. auris*	[72]
YSD1028	H99	*C. neoformans*	[73]

593

594

595 **Table 2:** Statistical significance values of survival of animals treated with antifungal drugs
596 compared to those not receiving treatment.
597

Fluconazole		**Caspofungin**	
Concentration	p-value	Concentration	p-value
1 mg/kg	0.54	1 mg/kg	0.8
4 mg/kg	0.63	2 mg/kg	0.015
16 mg/kg	0.68	4 mg/kg	0.0022
32 mg/kg	0.27		
64 mg/kg	0.13		

598
599

600 **Figure Captions**

601 **Figure 1: *M. sexta* caterpillars are susceptible to infections with *C. albicans*. a)** *M. sexta*
602 fifth instar caterpillar prior to injection weighing ~2 g. **b)** 24 hours post injection, the dead
603 animal on the left has lost colour and rigor compared to the live animal on the right. **c)**
604 Survival curves of animals infected with *C. albicans* SN95 or SC5314. Killing occurs in a
605 dose-dependent manner at 10^6 and 10^7 yeast cells per animal. **d)** Survival curves of animals
606 infected with *C. albicans* mutants with attenuated virulence phenotypes in mice or epithelial
607 cell models. The *hog1*Δ/Δ and *cka2*Δ/Δ mutants exhibit attenuated virulence, while virulence
608 of the *ahr1*Δ/Δ mutant is comparable to the wild type. The *hog1*Δ/Δ mutant differs
609 significantly from the wild type (p=0.0001). The complemented strain *hog1/hog1::HOG1*
610 kills *M. sexta* at a level comparable to that of the wild type strain (p=0.21). The *cka2*Δ/Δ
611 mutant is significantly less virulent than the wild type (p=0.00021), while the complemented
612 strain *cka2/cka2::CKA2* is not (p=0.054). Virulence of the *ahr1*Δ/Δ mutant and the
613 complemented strain *ahr1/ahr1::AHR1* does not differ significantly from the wild type
614 (*ahr1*Δ/Δ p=0.72; *ahr1/ahr1::AHR1* p=0.38).

615 **Figure 2: Elevated temperatures increase susceptibility of *M. sexta* to *C. albicans*. a)** *M.*
616 *sexta* caterpillars succumb to infection with the laboratory strain SC5314 in a dose-dependent
617 manner at 37˚C but less inoculum is required than for infections at 25˚C. **b)** The weight of
618 caterpillars infected with 10^4 cells per animal was comparable to that of caterpillars injected
619 with 1x PBS, while animals infected with 10^5 cells showed a significant reduction in weight.
620 **c)** Attenuated virulence of the *hog1*Δ/Δ mutant is retained at 37˚C. **d)** Animals infected with
621 the mutant strain gain significantly more weight than those infected with the wild type strain.
622 Infection with the complemented strain results in a comparable lack of weight gain.

623 **Figure 3: Fungal burden in faeces and hemolymph increases throughout the course of**
624 **infection.** Colony forming units per gram faeces **(a)** and 100 µl hemolymph **(b)** in the
625 *hog1*Δ/Δ mutant (YSD883), the wild type (SN95) and the control animals. Counts increase
626 over time in the wild-type infected animals but not the ones injected with the *hog1*Δ/Δ mutant
627 or PBS. **c)** Weight increases in animals infected with *hog1*Δ/Δ comparable to those injected
628 with PBS, while animals injected with the wild type *C. albicans* strain experience reduced
629 weight gain. The peak and drop in weight observed in the *hog1*Δ/Δ and PBS groups, marked
630 with a ⊙ is coinciding with the onset of pupation. This 'pupation drop' is due to the animals
631 refraining from food upon entering the early stages of pupation.

632 **Figure 4: Antifungal efficacy testing of fluconazole and caspofungin.** Groups of ten *C.*
633 *albicans* infected caterpillars were treated with increasing doses of antifungal drug. a)
634 Survival improves upon treatment with fluconazole, while weight remains largely stagnant in
635 surviving animals (b). c) Caspofungin treatment has a positive effect on *M. sexta* survival and
636 weight (d). Weight data were not analysed for significance due to the lack of animals in the
637 no treatment group.

638 **Figure 5: Caterpillars are susceptible to common yeast pathogens.** Groups of ten animals
639 were infected with increasing doses of yeasts and survival was recorded daily. Caterpillars
640 are not susceptible to *S. cerevisiae* or *M. pulcherrima* but infections with *C. neoformans*, *C.*
641 *glabrata*, *C. auris* and *C. albicans* result in significantly reduced survival rates.

642 **Figure 6: Caterpillar weight as a measure of virulence.** Recording caterpillar weight daily
643 revealed that yeast infections affect weight regardless of mortality rates. Daily weight
644 measures were compared to that of PBS-injected animals and statistical significance assessed
645 as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

646

647 **Figure S1: Caterpillar growth is comparable between standard colony temperature and**
648 **host body temperature.** Groups of ten animals were kept on standard diet at 25˚C and 30˚C
649 **(a)** or 25˚C and 37˚C **(b)** for four days. Animals were scored daily for survival and weight as
650 a measure of development. Within these parameters, caterpillar development is comparable
651 across all three temperatures.

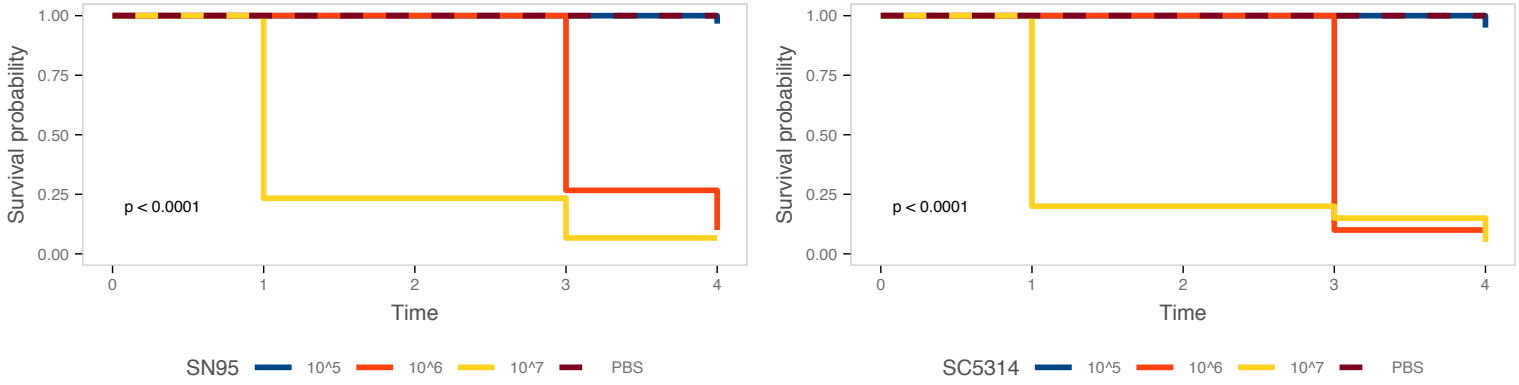
652 **Figure S2: Heat-killed *C. albicans* cells do not affect caterpillar survival or weight gain.**
653 Groups of ten animals were injected with either live *C. albicans* wild-type YSD85 cells, heat-
654 killed cells, or PBS. a) Only live yeast cells kill caterpillars. b) The weight gain in animals
655 infected with heat-killed *Candida* cells is comparable to that of animals injected with PBS.

Figure 1 - Lyons et al.

a)

b)

c)



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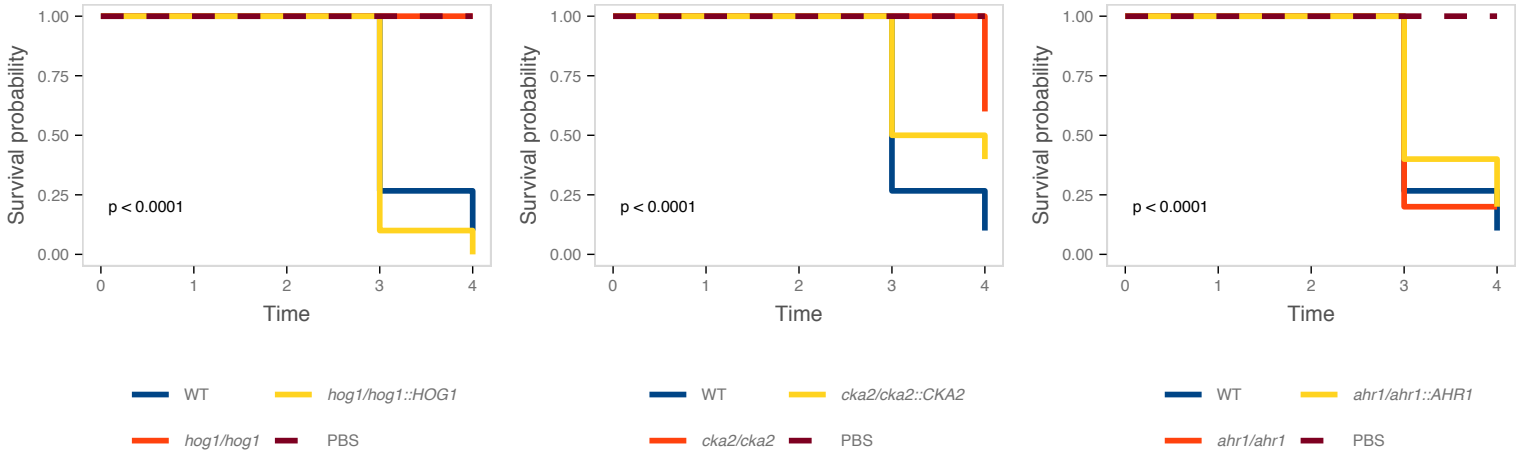


Figure 2 - Lyons et al.

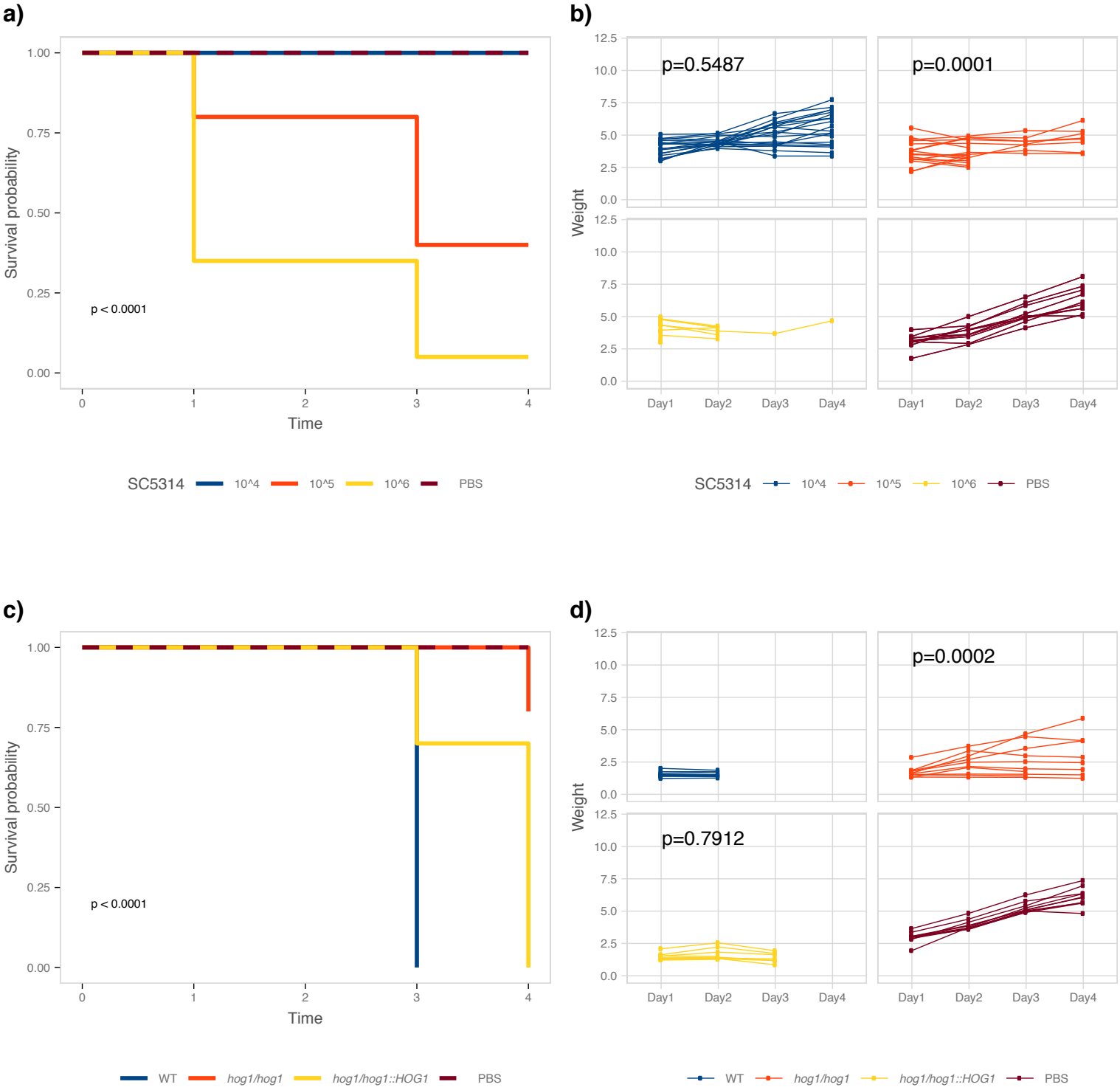
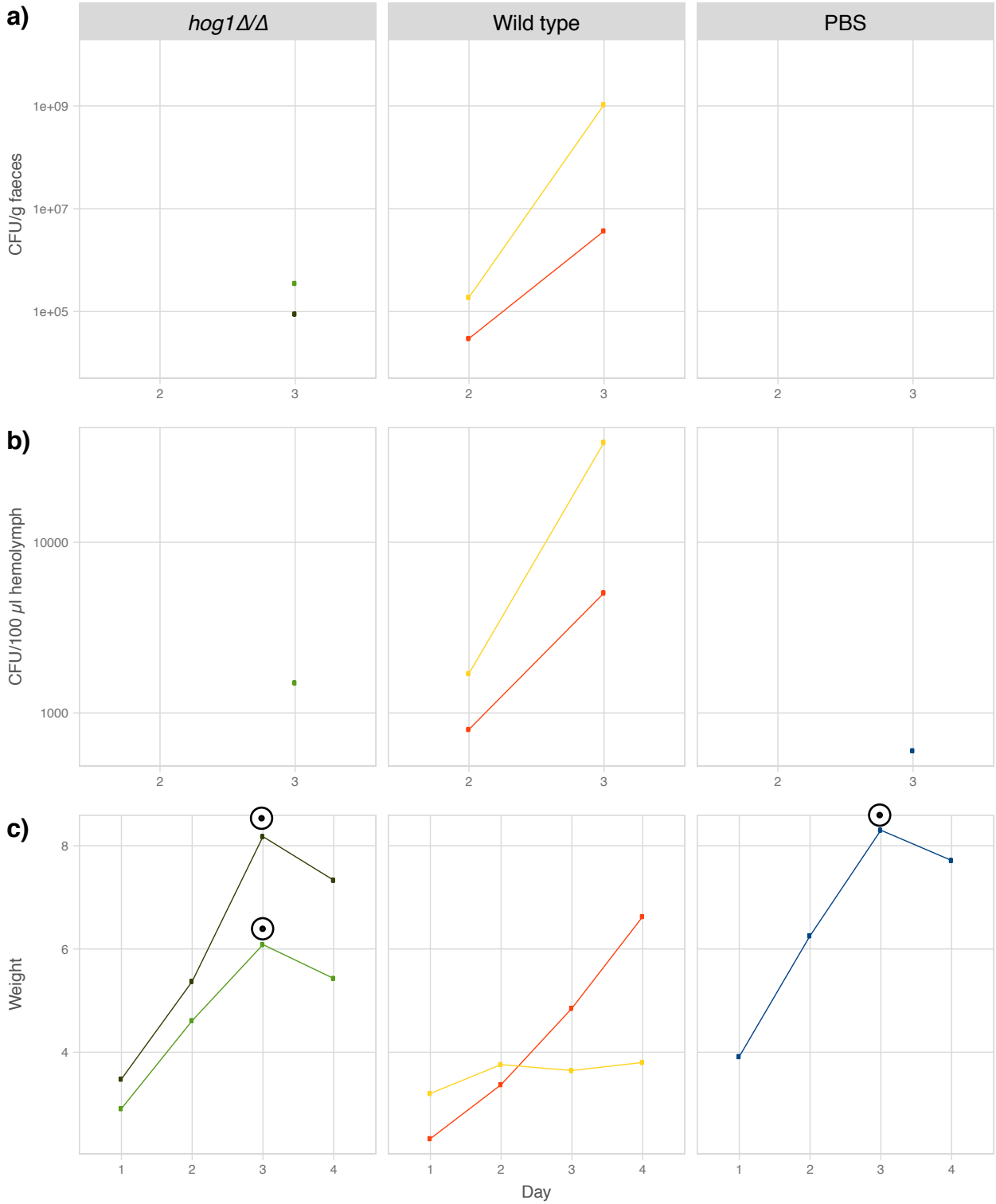


Figure 3 - Lyons et al.



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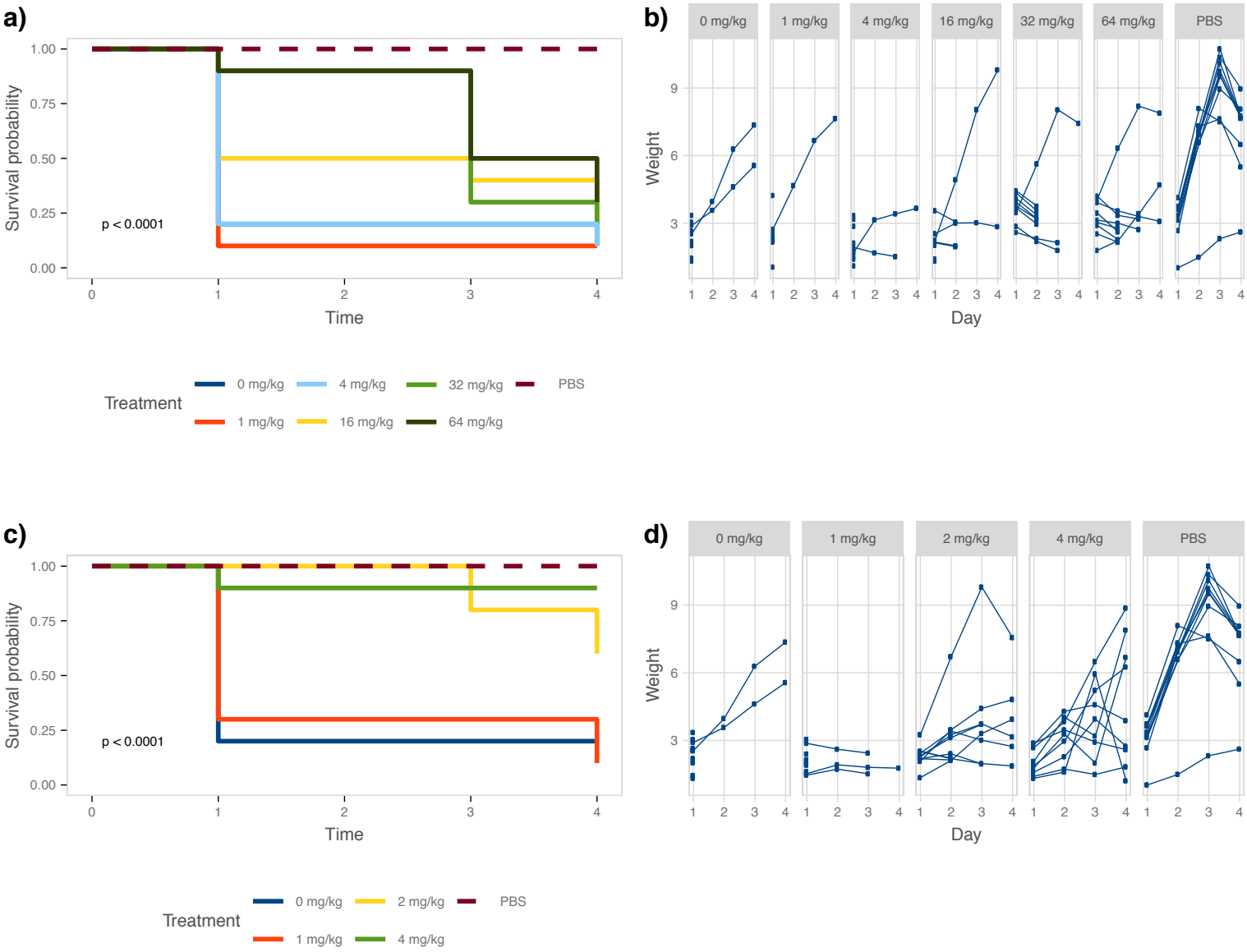

Figure 5 - Lyons et al.

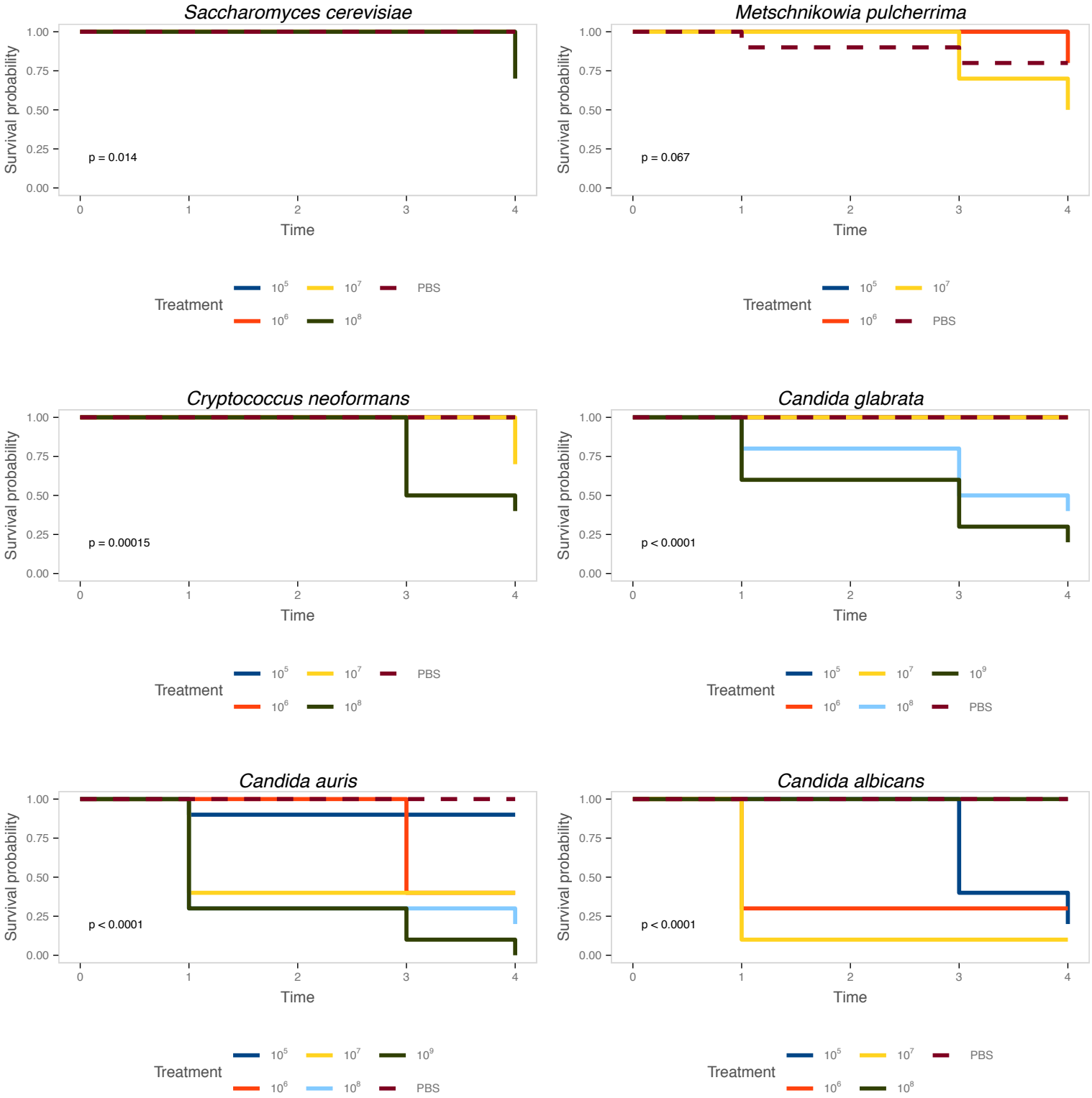

Figure 6 - Lyons et al.

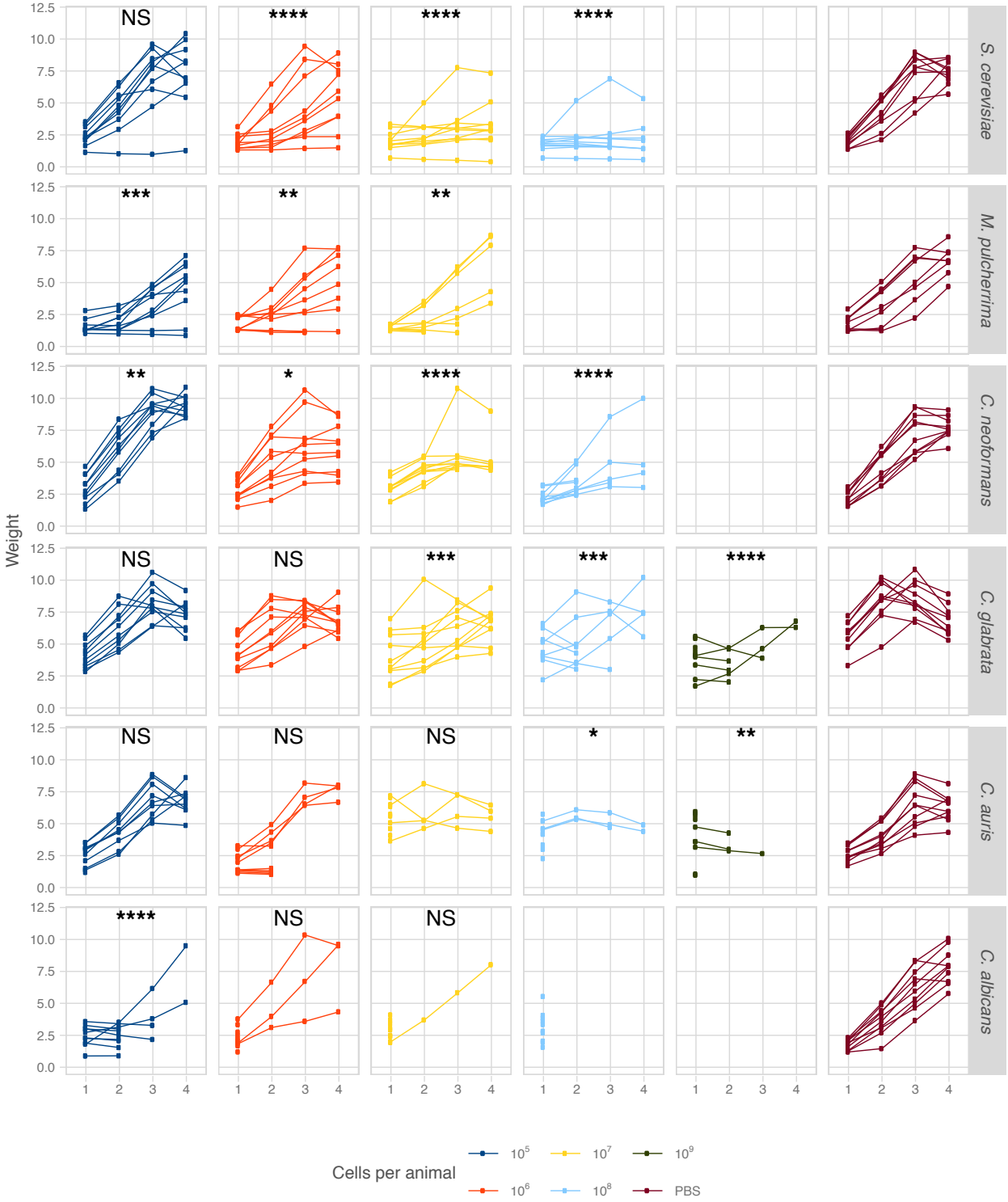

Figure S1 - Lyons et al.

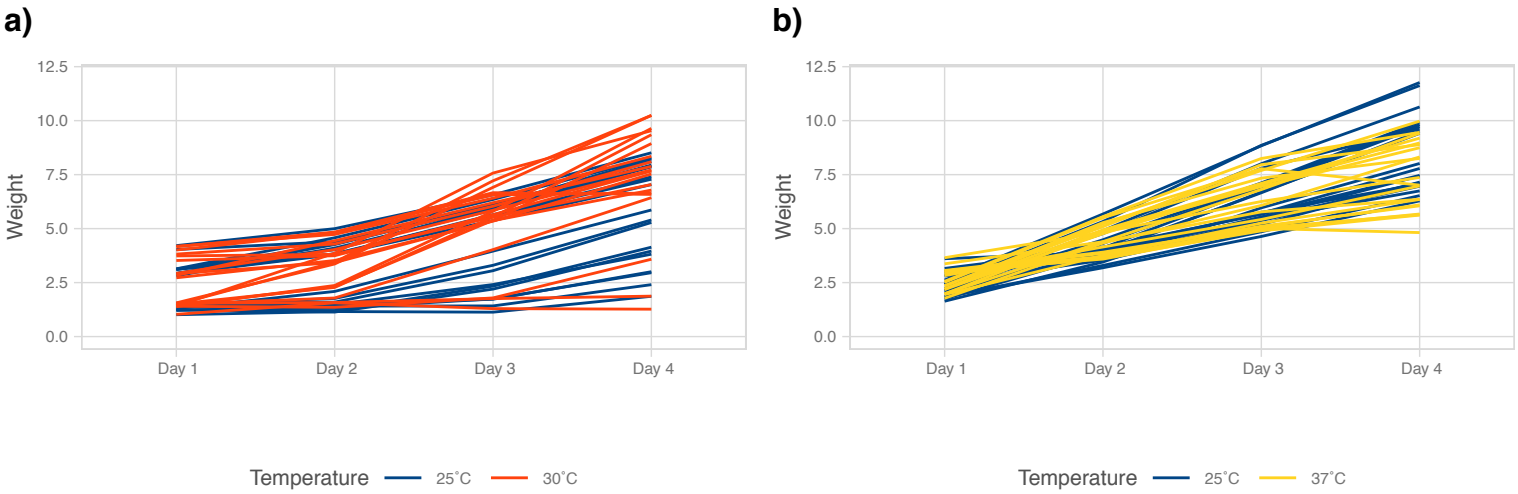
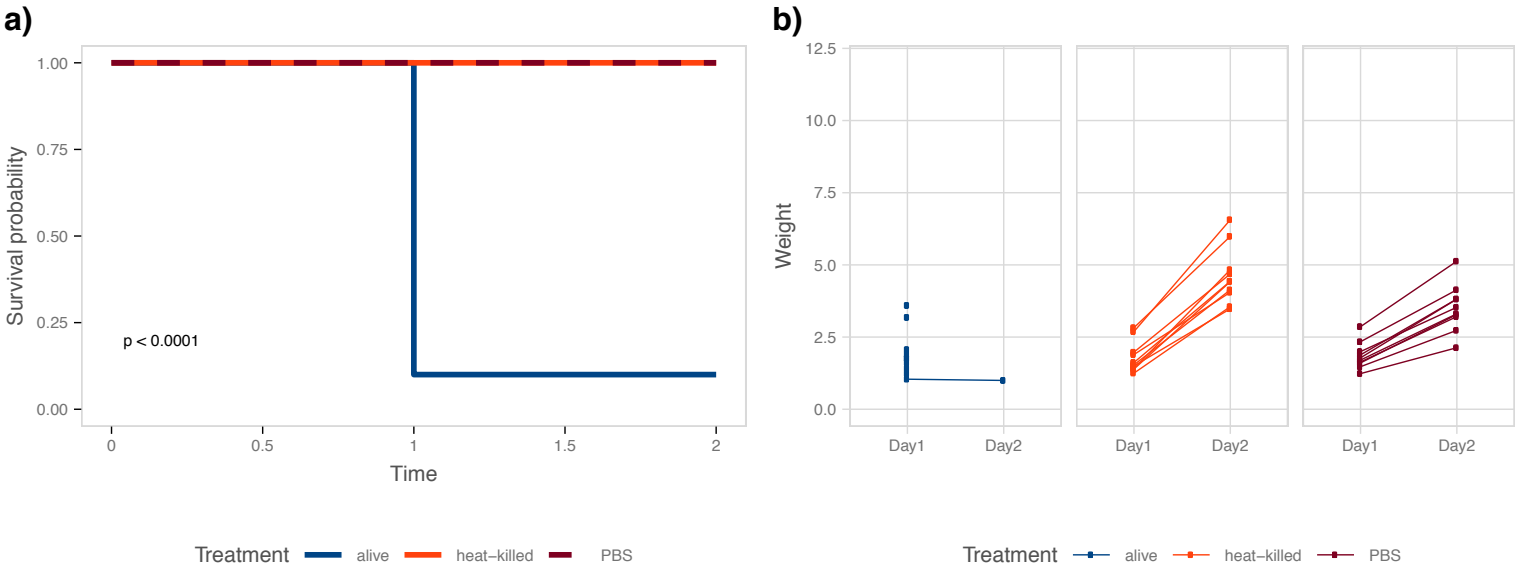
Figure S2 - Lyons et al.