Tobacco Hornworm (*Manduca sexta*) caterpillars as a novel host model for the study of fungal virulence and drug efficacy

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Abstract

The two leading yeast pathogens of humans, *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, efficacy of antifungal drugs, and host response pathways. While this is commonly done in mammalian models, which are afflicted by ethical and practical concerns, invertebrate models, such as wax moth larvae and nematodes have been introduced over the last two decades. To complement existing invertebrate host models, we developed fifth instar caterpillars of the Tobacco Hornworm moth *Manduca sexta* as a novel host model. These caterpillars can be maintained at 37˚C, are suitable for injections 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*. Importantly, fungal burden can be assessed daily throughout the course of infection in a single caterpillar's faeces and haemolymph. Infected caterpillars can be rescued by treatment with antifungal drugs. Notably, these animals are large enough for weight to provide a reliable and reproducible measure of fungal disease. *M. sexta* caterpillars combine a suite of parameters that make them suitable for the study of fungal virulence.

Keywords: *Manduca sexta*,
caterpillar,
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Saccharomyces,
Metschnikowia,
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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*, account for ~1,400,000 life-threatening infections world-wide with mortality rates of up to 70%[1,2]. Candidaemia, most frequently 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, candidaemia incidence rates are on the rise. Within less than ten years, they increased by 36%[3]. Although cryptococcosis incidence rates are on the decline in North America, cryptococcosis as an AIDS-defining illness is responsible for 15% of all AIDS-related deaths world-wide[3,4]. This dire situation is further confounded by the emergence of drug resistant yeast species, such as *C. glabrata* and *C. auris*. Patients at risk of developing invasive candidaemia are often prophylactically treated with fluconazole and echinocandins as a first line defence strategy[5]. Yet, *C. glabrata*, the most common non-*albicans Candida* species associated with nosocomial blood stream infections[6], is intrinsically less susceptible to azole drugs and acquires resistance to echinocandins rapidly[7]. The rapid global spread of multi-drug resistant *C. auris* has further exacerbated the threat posed by fungal infections. *C. auris* was first reported in 2009 in Japan[8]. In 2015, *C. auris* arrived in Europe causing an outbreak involving 72 patients in a cardio-thoracic hospital in London[9]. *C. auris* outbreaks have been reported from South Korea, India, Spain, Columbia, Switzerland, Germany, Israel, Kuwait, and Oman[10]. 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 candidaemia. In addition to the unacceptably high burden on human health, fungal infections substantially increase health care costs. Treatment of candidaemia requires extended hospitalisation, resulting in additional costs of up to $45,000 in adult patients or up to $119,000 in paediatric patients[11].

It is thus imperative to investigate fungal virulence and host response mechanisms. This is traditionally done in mammalian models. The most frequently deployed models include the mouse tail vein infection model for systemic candidaemia, the mouse gastrointestinal infection model of candidaemia, the mouse *Candida* vaginitis model[12], the mouse inhalation model of cryptococcosis[13], the rabbit chronic cryptococcal meningitis model[14], and the rabbit *Candida* keratitis model[15]. While mammalian models combine a

82 number of features that make them particularly amenable for the study of fungal diseases,
83 such as susceptibility, availability of knock-out mutants, and comparable histology to human
84 disease, using mammals is ethically controversial, economically challenging, and requires
85 extensive board certifications and documentations.

86 In an effort to reduce the usage of mammalian hosts, alternative invertebrate models
87 have been developed and successfully used over the past two decades. The most commonly
88 employed invertebrate species include the nematode *Caenorhabditis elegans*, the fly
89 *Drosophila melanogaster*, and larvae of the Greater Wax moth *Galleria mellonella*. All three
90 can be easily maintained in the laboratory, are much less expensive than mice or rabbits, and
91 have been used for the study of diverse yeast pathogens, such as *C. neoformans*[16,17], *C.*
92 *albicans*[17-19], *C. parapsilosis*[19-21], *C. glabrata*[20,22]. Of note, invertebrate models differ in their
93 applicability and the best suitable model should be carefully selected[23]. Unlike mammalian
94 models, these invertebrates do not have adaptive immunity but all share components of the
95 innate immune system[24,25], some of which are conserved with mammals. This includes the
96 Toll-like receptors found in the fly[26] and the homolog of the MKK3/6 kinase in the
97 nematode[27]. Ironically, it is the Toll-like receptors that protect flies from infections with *C.*
98 *neoformans*[28], *C. albicans*[29], and *C. glabrata*[30] and the MKK3/6 homolog SEK-1 protects the
99 nematode from bacterial invaders[27]. Thus, to increase susceptibility of flies and nematodes to
100 fungal pathogens, Toll and *sek-1*[31] mutants are required. A key limitation for the study of
101 human pathogens, is the inability of nematodes and flies to survive human body temperature.
102 Only *Galleria* can withstand 37˚C[17]. Due to their long-standing history as eukaryotic models,
103 well-curated genomes and genome databases exist for the nematode and the fly. Although the
104 *Galleria* genome has been announced very recently[32], detailed analyses and annotations are
105 still outstanding. Delivery of an exact inoculum of fungal cells is crucial when comparing
106 mutant and wild type yeast strains, neither fly nor nematode permit routine direct injection.
107 *Galleria* can be directly injected with a defined cell number. Extensive stock collections
108 provide fly and nematode strains, while until recently, *Galleria* larvae had to be purchased
109 from fishing shops. Now, UK-based TruLarv is selling research grade larvae.

110 Tobacco Hornworms are most commonly encountered in the southern United States,
111 where they feed on solanaceous plants and are thus considered a plant pest. As an insect
112 model with a long history in research, *Manduca sexta* has yielded important insights into
113 flight mechanisms, nicotine resistance, hormonal regulation of development, metamorphosis,

114 antimicrobial defences, and bacterial pathogenesis. *M. sexta* laboratory stocks have been
115 derived from animals collected in North Carolina, USA[33] and have been maintained in
116 laboratories on both sides of the Atlantic for several decades. *M. sexta*'s research portfolio
117 includes a draft genome sequence that has been complemented with tissue-specific
118 transcriptomic analyses[34], numerous successful applications of RNAi[35-39], and protocols for
119 the efficient extraction of hemocytes for down-stream analyses[40] of the animal's innate
120 immunity[41]. Despite its versatility, *M. sexta* has yet to be explored for its suitability as a host
121 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 were tested for their
124 ability to live at 37˚C, their susceptibility to different yeast species, and the reproducibility of
125 *C. albicans* mutant phenotypes obtained in mice virulence studies. Indeed, the caterpillars
126 grow at 37˚C while maintaining susceptibility to the leading yeast pathogens *C. albicans*, *C.*
127 *neoformans*, and emergent *C. auris*. Specific *C. albicans* mutants are just as attenuated in
128 their virulence in *M. sexta* as they are in mice. To expand *M. sexta's* applicability as a host
129 model, we developed an infection protocol that permits screening of fungal burden
130 throughout the course of infection in a single animal and uses weight as a proxy measure for
131 virulence in addition to survival. *M. sexta* can furthermore be used to test efficacy of common
132 antifungal drugs and to query the host transcriptional response to systemic yeast infections.
133 Our results define *M. sexta* characteristics that recommend these caterpillars as a non-
134 mammalian host model for the study of fungal virulence with susceptibility to different yeast
135 species.

136 **Materials and Methods**

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

138 The University of Bath colony has been in continuous culture since 1978 without the
139 addition of animals from elsewhere. Bath's genetic stock was derived from animals from the
140 Truman-Riddiford laboratories at the University of Washington in Seattle, USA. Their
141 animals date back to the ones originally collected in North Carolina in 1976[33].

142 **Caterpillar maintenance 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 'Food preparation') at a constant temperature of 25˚C with 50%
146 humidity and 12 hours light/dark cycles. Three days prior to fungal inoculations, animals
147 were shifted to a formaldehyde-free diet as the compound is toxic to non-methylotrophic
148 yeast.

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

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

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

162 To measure fungal burden in caterpillar faeces and haemolymph, six animals were
163 injected with 100 µl of either 1x PBS or 10^6 cells of the wild type YSD89 or the *hog1* mutant
164 strain YSD883 and kept at 37˚C. On day 1, two animals were selected from each group.
165 These animals were weighted and their haemolymph and faeces collected daily throughout
166 the course of infection. To collect haemolymph, animals were first kept on ice for 15 minutes.
167 The 'horn' was then surface sterilised with 70% ethanol and its top 1-2 mm clipped with a
168 pair of micro scissors. Haemolymph was collected in a pre-chilled 1.5 ml Eppendorf tube and
169 cooled immediately to reduce polymerisation and melanisation. One faecal pellet was
170 collected daily with sterile forceps, weighted and suspended in 500 µl 1x PBS. Prior to
171 diluting, the suspension was thoroughly vortexed for 10 seconds, and centrifuged for 5
172 seconds using a table top centrifuge to separate faecal matter. To quantify fungal burden,
173 haemolymph and faecal samples were plated either directly onto YPD-agar with Kanamycin

174 50 µg/ml or in ten-fold serial dilutions. Agar plates were incubated at 30˚C for 48 hours and
175 colonies counted.

176 To assess the efficacy of commonly used antifungal drugs, animals were infected with
177 10^7 cells of YSD85 or 100 µl PBS and treated with increasing doses of fluconazole and
178 caspofungin (Sigma Aldrich, Inc.) as indicated. Drugs were injected 30 minutes post-
179 infection with an ethanol-sterilized Hamilton syringe in a total volume of 10 µl per animal.
180 Caterpillars were weighed and scored for survival on the day of injection and the following
181 three days.

182 **Statistical analyses**

183 Survival plots were made using the survminer R package (https://CRAN.R-
184 project.org/package=survminer), and differences were evaluated using the Kaplan-Meier
185 method. Weight and fungal burden were plotted using ggplot2[42] and weight differences were
186 evaluated using linear models with day post-inoculation and the interaction between
187 treatment and days post infection as fixed effects and individual as a random effect using
188 nlme (https://CRAN.R-project.org/package=nlme). Upset plots visualising gene expression
189 profiles were made using the UpSetR package[43]. All analyses were conducted in RStudio
190 version 1.1.442.

191 **RNA sequencing and gene expression analysis**

192 Five animals were injected with 100 µl 1x PBS or 10^6 yeast cells of the wild type
193 strain SN95 (YSD89) and maintained on a 12-hour light/dark cycle at 37˚C for 24 hours.
194 Three animals were then randomly selected for transcriptomic analyses of their midguts. In
195 preparation for midgut extraction, animals were surface sterilized and tissue extracted and
196 washed as described before[34]. Midgut RNA was extracted using TRIzol reagent (Invitrogen,
197 Inc.), quantified using the Agilent 2100 Bioanalyzer system and used to generate RNA-seq
198 libraries (strand-specific, paired end) using the TruSeq RNA sample prep kit (Illumina).
199 150 nucleotides of the sequence were determined from both ends of each cDNA
200 fragment using the HiSeq 2500 platform (Illumina). An average of 83.79 million read pairs
201 were obtained for each sample (range: 51.4 M to 109.8 M read pairs). Sequencing reads were
202 aligned to the reference *Manduca sexta* whole genome assembly v1.0
203 (https://i5k.nal.usda.gov/Manduca_sexta) using HISAT2[44] and alignment files were used to
204 generate read counts for each gene. Statistical analysis of differential gene expression was

205 performed using the DEseq2 package from Bioconductor[45]. A gene was considered
206 differentially expressed if the FDR-value for differential expression was less than 0.10.
207 In order to compare the transcriptional response to *C. albicans* infection by *M. sexta* to that of
208 a mouse, we generated a blast reference database using the peptide sequences for 67,960
209 proteins from the mouse genome (GRCm38) available from Ensembl
210 (https://useast.ensembl.org). We downloaded the peptide sequences for 27,403 proteins from
211 the *M. sexta* database (OGS v2.0) available from the National Agricultural Library
212 (https://i5k.nal.usda.gov/Manduca_sexta)[34] provided by the United States Department of
213 Agriculture. We computed peptide sequence similarity between the *M. sexta* proteins and the
214 mouse proteins using the blastp local alignment search tool (ncbi-blast+ v2.8.1)[46]. For each
215 *M. sexta* protein, we had multiple hits that were ranked based on the e-value computed by the
216 blastp search tool. For the list of differentially expressed *M. sexta* genes from each
217 comparison, we extracted the mouse orthologs with e-values smaller than e^{-20}. This list of
218 orthologs was then compared to differential expression lists based on RNA-seq analysis of
219 kidneys[47], tongues[48], and vaginas[49] from *C. albicans* infected mice. Gene lists were compared
220 using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html). RNAseq data are
221 available from NCBI's Short Read Archive (###).

222 **Results**

223 ***M. sexta* caterpillars are susceptible to the leading fungal pathogen *C. albicans* at 37˚C**

224 We first aimed to determine if *M. sexta* fifth instar caterpillars (Fig. 1a), reared and
225 maintained at standard conditions, are indeed susceptible to *C. albicans*. To do so, groups of
226 ten animals were infected with increasing doses of the widely used *C. albicans* laboratory
227 strains SC5314 and SN95[50]. Animals were kept at 25˚C, their standard maintenance
228 temperature, and scored for survival on three consecutive days. Dead animals differ from live
229 ones in that their bodies go limp and turn brown-green in colour, which is in stark contrast to
230 the vivid turquoise of live animals (Fig. 1b). Caterpillars infected with *C. albicans*
231 succumbed in a dose-dependent manner. Both *C. albicans* strains killed *M. sexta* efficiently at
232 inocula of 10^6 or 10^7 cells per animal (Fig. 1c). To assess if survival measures in caterpillars
233 are comparable to those obtained in the current gold standard, the murine model of systemic
234 candidaemia, we tested *C. albicans* mutants with published phenotypes of either attenuated
235 virulence, such as the *hog1*Δ/Δ[51] and *ahr1*Δ/Δ[52] mutants, or wild-type levels of virulence,
236 such as *cka2*Δ/Δ[53]. Cross-species virulence levels are comparable for Hog1, which is just as

237 essential for virulence in caterpillars as it is in mice. Ahr1, while required for virulence in
238 mammals, appears to be dispensable for virulence in caterpillars. Cka2 is not required to
239 establish systemic infections in mammals but is in caterpillars (Fig. 1d).

240 Given the importance of temperature for fungal virulence, we queried if *M. sexta*
241 retained their susceptibility to *C. albicans* at human body temperature of 37˚C. Temperature
242 itself does not affect caterpillar survival or development (Fig. S1) but animals are ten times
243 more susceptible to infections with *C. albicans* at 37˚C than they are at 25˚C (Fig. 2a). At
244 37˚C, 10^6 *C. albicans* cells per animal lead to 100% mortality on day 4, while 10^7 cells are
245 required for the same outcome at 25˚C (Fig. 1b). To exclude the possibility that mortality is
246 due to starvation rather than the outcome of a host-pathogen interaction, we infected
247 caterpillars with live and heat-killed *C. albicans* wild-type cells and kept them at 37˚C. Only
248 live cells, but not heat-killed yeast cells, killed the caterpillars suggesting that killing is not
249 due to nutritional limitations (Fig. S2). Demonstrating susceptibility of *M. sexta* caterpillars
250 to *C. albicans* lent support to them as an alternative host model for the study of fungal
251 virulence and emphasized the need for additional measures of fungal virulence. To add
252 granularity to fungal virulence data collected from *M. sexta*, we complemented measures of
253 survival with quantifications of weight and fungal burden throughout the course of infection.
254 To collect weight data, caterpillars were weighed prior to infection and then daily throughout
255 the course of infection. Weight gain in animals infected with a low dose of 10^4 cells did not
256 significantly differ from those injected with 1x PBS but caterpillars infected with10^5 cells per
257 animal exhibited significant weight loss. Too few animals survived infection with 10^6 cells to
258 allow for a meaningful comparison (Fig. 2b). After establishing susceptibility of *M. sexta* to
259 *C. albicans* at 37˚C, we aimed to validate the attenuated virulence phenotype of the *hog1*Δ/Δ
260 mutant strain. To test this, caterpillars were infected with 10^6 cells per animal with the wild
261 type strain, the *hog1*Δ/Δ deletion mutant, the *hog1/hog1::HOG1* complementation strain and
262 compared to the control group injected with 1x PBS. The *hog1*Δ/Δ mutant is less virulent
263 than the wild type (Fig. 2c). Animals infected with the *hog1*Δ/Δ mutant strain gained
264 significantly more weight than those infected with the wild type strain, while infection with
265 the complemented strain lead to a comparable lack in weight gain (Fig. 2d).

266 **Measurements of fungal burden in faeces and haemolymph obtained from a single**
267 **animal throughout infection provides a useful measure of virulence**

268 To further expand the applicability of *M. sexta* caterpillars as a host model for fungal
269 infections, fungal burden in the haemolymph and faeces was quantified daily throughout the
270 course of infection. Since the collection of haemolymph or faeces does not necessitate killing
271 the animal, data could be collected daily throughout the infection for the same caterpillar.
272 Animals infected with the wild type and the *hog1*Δ/Δ mutant were compared to control
273 animals injected with 1x PBS only and fungal burden measured as colony-forming units
274 (CFUs) in faeces and haemolymph in two animals per group. Yeasts were detected in the
275 faeces and haemolymph of animals infected with the wild type on day 2 and CFU counts
276 increased on day 3 (Fig. 3a, b). In animals infected with the *hog1*Δ/Δ mutant, we detected low
277 CFU counts on day 3 in faeces and haemolymph. Even fewer CFUs were detected in the
278 haemolymph of animals injected with PBS on day3. Consistent with the observed increase in
279 fungal burden in wild-type infected animals, indicative of a fungal infection, these animals
280 displayed reduced weight gain while *hog1*Δ/Δ infected animals gained weight at a similar rate
281 as the control animals (Fig. 3c). Thus, measurements of fungal burden, that can be obtained
282 from the same animal throughout the course of infection, provide a valuable parameter for the
283 study of fungal virulence.

284 **Caterpillars are suitable for antifungal drug treatment studies**

285 The currently available armamentarium of antifungal drugs is limited and drugs are
286 often lacking in efficacy. The ability to study drug efficacy and mode of action in a host
287 model are thus pertinent to the development of novel antifungal drugs. To determine *M.*
288 *sexta*'s suitability for drug efficacy testing, we recorded survival and weight of animals
289 infected with the *C. albicans* wild-type strain SC5314 that were treated with increasing doses
290 of two common antifungals, fluconazole and caspofungin (Fig.4). Treatment with fluconazole
291 or caspofungin resulted in overall improved survival and weight gain. Animals treated with 2
292 mg/kg and 4 mg/kg of caspofungin survived significantly better than those without treatment
293 or those that only received 1 mg/kg of caspofungin. Pair-wise comparisons between different
294 drug concentrations and the untreated control animals yielded no statistical significance for
295 the fluconazole-treated group of caterpillars (Table 2).

296 ***M. sexta* caterpillars are broadly susceptible to different yeast pathogens**

297 While *C. albicans* undoubtedly is a leading fungal pathogen of humans, clinical
298 manifestations of fungal infections are not limited to *C. albicans*. To broaden *M. sexta*'s

299 applicability, we sought to quantify the caterpillars' susceptibility to other fungal pathogens.
300 To this end, animals were also infected with wild type strains of *C. neoformans*, *C. auris*, and
301 *C. glabrata*. Type strains of *Saccharomyces cerevisiae*, the baker's or brewer's yeast and
302 *Metschnikowia pulcherrima*, a yeast inhabiting fruits and flowers[54] served as reference points
303 for attenuated virulence. Animals were infected with increasing doses of yeast cells starting at
304 10^5 cells per animal and up to 10^9 cells per animal. Groups of ten animals per yeast dose and
305 species were then screened for survival and weight daily (Figs. 5 and 6). Notably, only
306 infections with pathogenic yeast species affected survival of caterpillars (Fig. 5). Infections
307 with *S. cerevisiae* or *M. pulcherrima* did not significantly reduce caterpillar survival.
308 Comparing survival amongst the pathogenic yeast species revealed *C. albicans* to be the most
309 severe. 10^7 *C. albicans* cells per animal result in 100% killing within in 24 hours. 10^9 *C. auris*
310 cells were required to kill all animals within four days and the same dose of *C. glabrata*
311 resulted in 75% killing. 10^8 *C. neoformans* cells were required to achieve 50% killing within
312 four days, comparable to *C. glabrata*. It should be noted that due to the high viscosity of the
313 cell suspension, we could not test higher concentrations than the ones stated here. In addition
314 to assessing survival, each surviving caterpillar's weight was recorded daily. Interestingly,
315 while infections with *S. cerevisiae* or *M. pulcherrima* did not kill caterpillars, infected
316 animals did not gain weight as effectively as uninfected control animals (Fig. 6). Infections
317 with *C. albicans* resulted in severely reduced weight gain, even at the lowest yeast dose
318 tested. Animals infected with *C. auris* and *C. glabrata* responded in a dose-dependent
319 manner, the higher the yeast dose, the more dramatic the weight loss. Notably, animals
320 infected with the lowest dose of *C. neoformans* grew better than PBS-injected control
321 animals. Increasing the yeast dosage, however, resulted in significantly reduced weight gain.
322 This bi-phasic pattern, resembling hormesis[55] in which a low dose of an environmental agent
323 is beneficial while a high dose is toxic, could be due to *C. neoformans*' immunogenic
324 capsule[56]. Caterpillars displayed variable degrees of susceptibility to different yeast
325 pathogens and with weight providing an additional measure of host damage. Thus, *M. sexta*
326 caterpillars are broadly applicable for the study of fungal virulence.

327 **_M. sexta_ transcriptional response profiles differ between uninfected and infected**
328 **animals**

329 As a proof-of-concept we recorded transcriptional profiles in three animals infected
330 with the wild-type strain and compared them to three uninfected control animals. To do so,

331 we collected the mid-gut, rinsed the tissue thoroughly, and extracted RNA, which was then
332 sequenced using Illumina' HiSeq 2500 platform. With the vast majority of reads mapping to
333 *M. sexta* rather than *C. albicans* (Fig. S3), animals infected with *Candida* displayed a
334 transcriptional profile different from that of uninfected animals (Fig. S4, Table S1). Infection
335 with the wild type strain elicited quantifiable transcriptional changes in the caterpillars. 157
336 *M. sexta* genes were up-regulated and 165 went down when compared to the PBS control
337 animals (Table S1).

338 Lastly, we compared our set of differentially expressed *M. sexta* genes to those
339 identified in three murine candidaemia studies. Here, tissue-specific responses in the
340 kidneys[47], tongues[48], and vaginas[49] were recorded for animals infected with *Candida* wild
341 type (Fig. 7). *M. sexta* genes up-regulated during infection and their homologs in all three
342 mouse tissues include serine peptidase inhibitors (Serpinb3c, Serpinb3b), a Ras-like protein
343 (Rit2), transmembrane proteases (Tmprss11a, Tmprss11f), a histidine decarboxylase (Hdc),
344 and a peptidoglycan recognition protein (Pglyrp3). *M. sexta* genes down-regulated in mouse
345 kidney and vagina include a muscle glycogen phosphorylase (Pygm), a bone morphogenetic
346 antagonist of the DAN family (Grem1), meprin 1 alpha (Mep1a), and calponin 1 (Cnn1).
347 Two of these genes are annotated as functioning in immune system processes (Grem1,
348 Pglyrp3) and three more are relevant for signalling and response to different stimuli (Rit2,
349 Pglyrp3, Pygm). Seven genes, however, are involved in protein metabolic processes
350 (Serpinb3c, Serpinb3b, Rit2, Tmprss11a, Tmprss11f, Grem1, Mep1a), suggesting this
351 fundamental process to be of importance in the defence against microbial attacks.

352 **Discussion**

353 Invertebrate host models have become a valuable alternative to mammalian hosts for
354 the study of fungal disease. When choosing the appropriate invertebrate model, care needs to
355 be taken and experimental parameters should be considered[57]. Here we developed *M. sexta*
356 caterpillars as a novel invertebrate model showing that they are naturally susceptible to
357 different human pathogenic yeast species, including the emergent *C. auris*. Unlike other host
358 models, these large caterpillars permit daily measures of fungal burden throughout the course
359 of infection in a single animal by either collecting faeces or haemolymph. *M. sexta* can be
360 maintained at 37˚C, is large enough to be injected with a specified yeast inoculum and for
361 weight to be a reliable measure of virulence. *C. albicans* mutant virulence phenotypes found
362 in mice can be replicated and yeast inocula required to elicit a response in caterpillars are

363 comparable to those used in the murine model. Additionally, the caterpillars permit study of
364 antifungal drug efficacy. These parameters commend this invertebrate species as a novel host
365 model for the study of fungal virulence.

366 This new model system allows for fungal burden to be monitored throughout the
367 course of infection in a single animal via CFU count. This is unlike any other experimental
368 system, where fungal burden is either an endpoint measure in the mouse kidney, in
369 homogenised wax moth larvae[58], nematodes[59], flies[60], or requires genetically modified
370 fluorescent yeast strains for microscopic imaging and analyses[61]. While we did detect very
371 low CFU counts in the PBS control 3 days post infection, we consider this a spurious finding
372 due to cross contamination as preliminary experiments of plating contents of haemolymph
373 and faeces of naïve animals did not detect any yeast growth (data not shown). As a
374 consequence, we amended the protocol to include changing gloves when handling animals of
375 different treatment groups.

376 When reviewing the weight data collected as part of our study, we noticed two
377 interesting aspects. First, while *S. cerevisiae* and *M. pulcherrima* do not affect caterpillar
378 survival, infections with either species led to reduced weight gain. The dichotomy between
379 survival and weight observed here, further emphasizes that fungal virulence comprises more
380 than a measure of survival. It appears that *M. sexta* would allow discrimination between
381 disease (weight) and death (survival) quantitatively adding further granularity to measuring
382 fungal virulence. Secondly, infections with *C. neoformans* resulted in increased caterpillar
383 weight gain at a low yeast dose but reduced weight gain at higher doses. This pattern
384 resembles the concept of hormesis often deployed by toxicologists to describe the response to
385 toxins, where exposure to a low dose is beneficial while a higher dose results in toxicity due
386 to overcompensation in response to disruption of homeostasis[62]. While tissue-specific
387 hormetic responses have been described in flies, where a virus-acquired cytokine delays
388 ageing[63], and examples of abiotic factors or signalling molecules affecting peas and aphid
389 infestation[64], immunity in plants[65,66], life-span in malaria-transmitting *Anopheles*[67], and larval
390 development in Black Cutworm[68], this example here could be the first involving a eukaryotic
391 host-pathogen relationship. The underlying factor remains to be elucidated but *C.*
392 *neoformans*' highly immunogenic capsule seems to be an excellent candidate[56].

393 Heat-killed *C. albicans* cells appear to be entirely non-pathogenic in *M. sexta*
394 caterpillars as neither survival nor weight are affected by inoculation with heat-killed yeast

395 cells. The lack of mortality in response to inoculation with heat-killed yeast cells indicates
396 that yeast viability and proliferation are required for pathogenesis and excludes the possibility
397 of death due to an allergic reaction in response to a large number of fungal cells. This appears
398 to differ from the responses of other host models to fungal pathogens. While susceptibility
399 was reduced, but still measurable, in *G. mellonella*[69] and the two-spotted cricket[70], heat-killed
400 *C. albicans* cells elicited 100% mortality in a sepsis-like murine model[71]. In mice, serum
401 levels of β-(1,3)-glucan levels were elevated in animals injected with heat-killed yeast cells
402 when compared to those infected with live cells. Indeed, heat inactivation leads to increased
403 exposure of β-(1,3)-glucan on the *C. albicans* cell surface[72] and β-(1,3)-glucan activates the
404 innate immune response in invertebrates and mammals[73]. Given the complexity of receptors
405 involved in recognition of fungal invaders[74] and the lack of a response in *M. sexta* and other
406 invertebrates indicates that they may not express all or identical receptors as mammals.

407 Measures of the host transcriptional response provide useful insights into the
408 relevance of specific defence mechanisms. Mouse transcriptional profiles in response to *C.*
409 *albicans* infections differ between different types of tissues[48,49,75], which allowed for the
410 identification of tissue-specific response mechanisms such as IL-17 signalling in oral
411 epithelia. Here, we detected numerous differentially expressed genes in the caterpillar midgut
412 when comparing animals infected with *C. albicans* to the PBS control. Given the size of *M.*
413 *sexta* caterpillars, which permits tissue-specific transcriptional analyses[34], this phenomenon
414 could be systematically investigated in caterpillars rather than mammalian hosts, who are
415 burdened by ethic concerns and economic challenges prohibiting large scale or time-course
416 studies. Furthermore, partial conservation of the transcriptional response to *C. albicans*
417 infections between insects and mammals offers the opportunity to identify and investigate
418 core eukaryotic response mechanisms.

419 In summary, *Manduca sexta* caterpillars expand the current repertoire of invertebrate
420 models for the study of fungal disease. They combine a suite of measures that commend it as
421 a new model system. Although, *M. sexta* genomic and transcriptomic analyses are currently
422 still in their infancy, we would expect that the Tobacco Hornworm's long history of being an
423 invaluable model for diverse facets of biology will lead to reliable tools in combination with
424 genetic tractability and protocols establishing the fungus' fate inside the caterpillar.

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Disclosure of interest

The authors report no conflict of interest.

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684 **Appendix – Food preparation**

685

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

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

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

696

697 **Tables**

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

Strain ID	Strain Name/genotype	Species	Source
YSD89	SN95	*C. albicans*	
YSD85	SC5314	*C. albicans*	
YSD883	*hog1*Δ/Δ	*C. albicans*	This study
YSD87	BWP17	*C. albicans*	[76]
YSD190	VIC84 (*cka2*Δ/Δ)	*C. albicans*	[77]
YSD192	VIC93 (*cka2/cka2::CKA2*)	*C. albicans*	[77]
YSD302	CAS12 (*ahr1*Δ/Δ)	*C. albicans*	[52]
YSD303	CAS13 (*ahr1/ahr1::AHR1*)	*C. albicans*	[52]
YSD304	RM1000	*C. albicans*	[78]
YSD305	JC52 (*hog1/hog1::HOG1*)	*C. albicans*	[78]
YSD306	JC50 (*hog1*Δ/Δ)	*C. albicans*	[78]
YSD790	YPS143	*S. cerevisiae*	[79]
YSD1448	NCYC2580	*M. pulcherrima*	[80]
YSD465	2001	*C. glabrata*	[81]
YSD1454	TA004-14	*C. auris*	[82]
YSD1028	H99	*C. neoformans*	[83]

699

700

701 **Table 2:** Statistical significance values of survival of animals treated with antifungal drugs
702 compared to those not receiving treatment.
703

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		

704
705

706 **Figure Captions**

707 **Figure 1: *M. sexta* caterpillars are susceptible to infections with *C. albicans*. a)** *M. sexta*
708 fifth instar caterpillar prior to injection weighing ~2 g. **b)** 24 hours post injection, the dead
709 animal on the left has lost colour and turgidity compared to the live animal on the right. **c)**
710 Survival curves of animals infected with *C. albicans* SN95 or SC5314. Killing occurs in a
711 dose-dependent manner at 10^6 and 10^7 yeast cells per animal. **d)** Survival curves of animals
712 infected with *C. albicans* mutants with attenuated virulence phenotypes in mice or epithelial
713 cell models. The *hog1*Δ/Δ and *cka2*Δ/Δ mutants exhibit attenuated virulence, while virulence
714 of the *ahr1*Δ/Δ mutant is comparable to the wild type. The *hog1*Δ/Δ mutant differs
715 significantly from the wild type (p=0.0001). The complemented strain *hog1/hog1::HOG1*
716 kills *M. sexta* at a level comparable to that of the wild type strain (p=0.21). The *cka2*Δ/Δ
717 mutant is significantly less virulent than the wild type (p=0.00021), while the complemented
718 strain *cka2/cka2::CKA2* is not (p=0.054). Virulence of the *ahr1*Δ/Δ mutant and the
719 complemented strain *ahr1/ahr1::AHR1* does not differ significantly from the wild type
720 (*ahr1*Δ/Δ p=0.72; *ahr1/ahr1::AHR1* p=0.38).

721 **Figure 2: Elevated temperatures increase susceptibility of *M. sexta* to *C. albicans*. a)** *M.*
722 *sexta* caterpillars succumb to infection with the laboratory strain SC5314 in a dose-dependent
723 manner at 37˚C but less inoculum is required than for infections at 25˚C. **b)** The weight of
724 caterpillars infected with 10^4 cells per animal was comparable to that of caterpillars injected
725 with 1x PBS, while animals infected with 10^5 cells showed a significant reduction in weight.
726 **c)** Attenuated virulence of the *hog1*Δ/Δ mutant is retained at 37˚C. **d)** Animals infected with
727 the JC50 mutant strain gain significantly more weight than those infected with the wild type
728 strain RM1000. Infection with the complemented strain JC52 resulted in a comparable lack of
729 weight gain.

730 **Figure 3: Fungal burden in faeces and haemolymph increases throughout the course of**
731 **infection.** Colony forming units per gram faeces **(a)** and 100 µl haemolymph **(b)** in the
732 *hog1*Δ/Δ mutant (YSD883), the wild type (SN95) and the control animals. Counts increase
733 over time in the wild-type infected animals but not the ones injected with the *hog1*Δ/Δ mutant
734 or PBS. **c)** Weight increases in animals infected with *hog1*Δ/Δ comparable to those injected
735 with PBS, while animals injected with the wild type *C. albicans* strain experience reduced
736 weight gain. The peak and drop in weight observed in the *hog1*Δ/Δ and PBS groups, marked

737 with a ⊙ is coinciding with the onset of pupation. This 'pupation drop' is due to the animals
738 refraining from food upon entering the early stages of pupation.

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

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

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

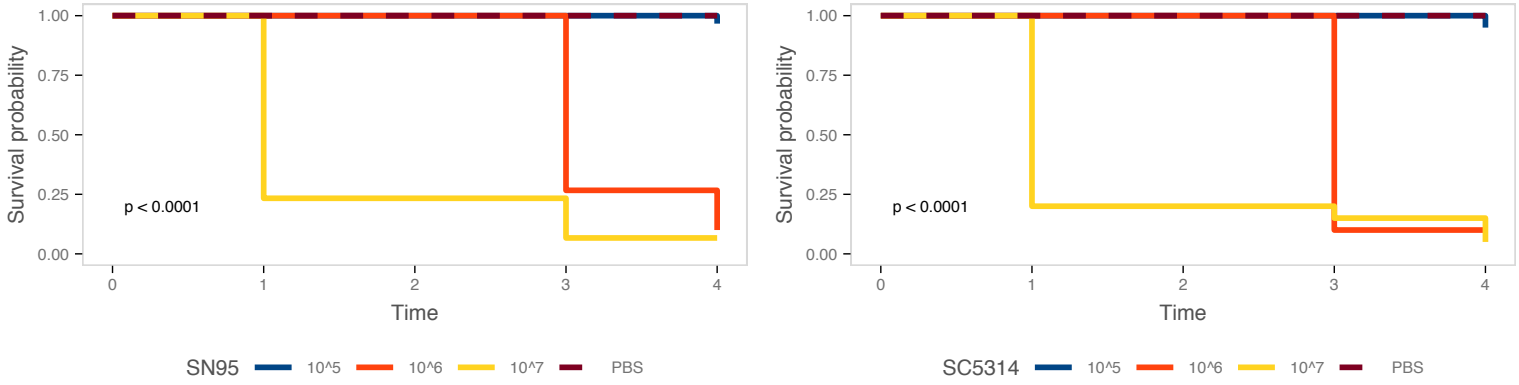
753 **Figure 7: Mouse homologs of *M. sexta* differentially expressed genes.** Numbers of
754 *Manduca* genes and their murine homologs that were up- or down-regulated in mouse vagina,
755 kidney or tongue and the overlap between the different data sets as depicted in a Euler
756 diagram. Note, in some cases the same mouse gene was the best BLAST match for several *M.*
757 *sexta* genes.

Figure 1 - Lyons et al.

a)

b)

c)



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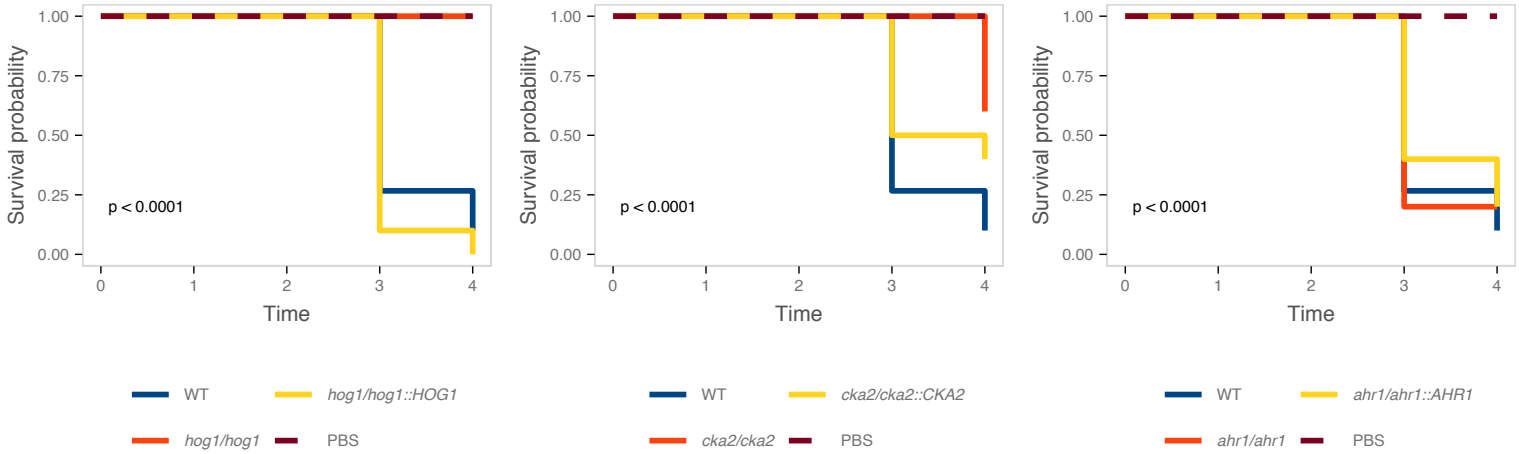


Figure 2 - Lyons et al.

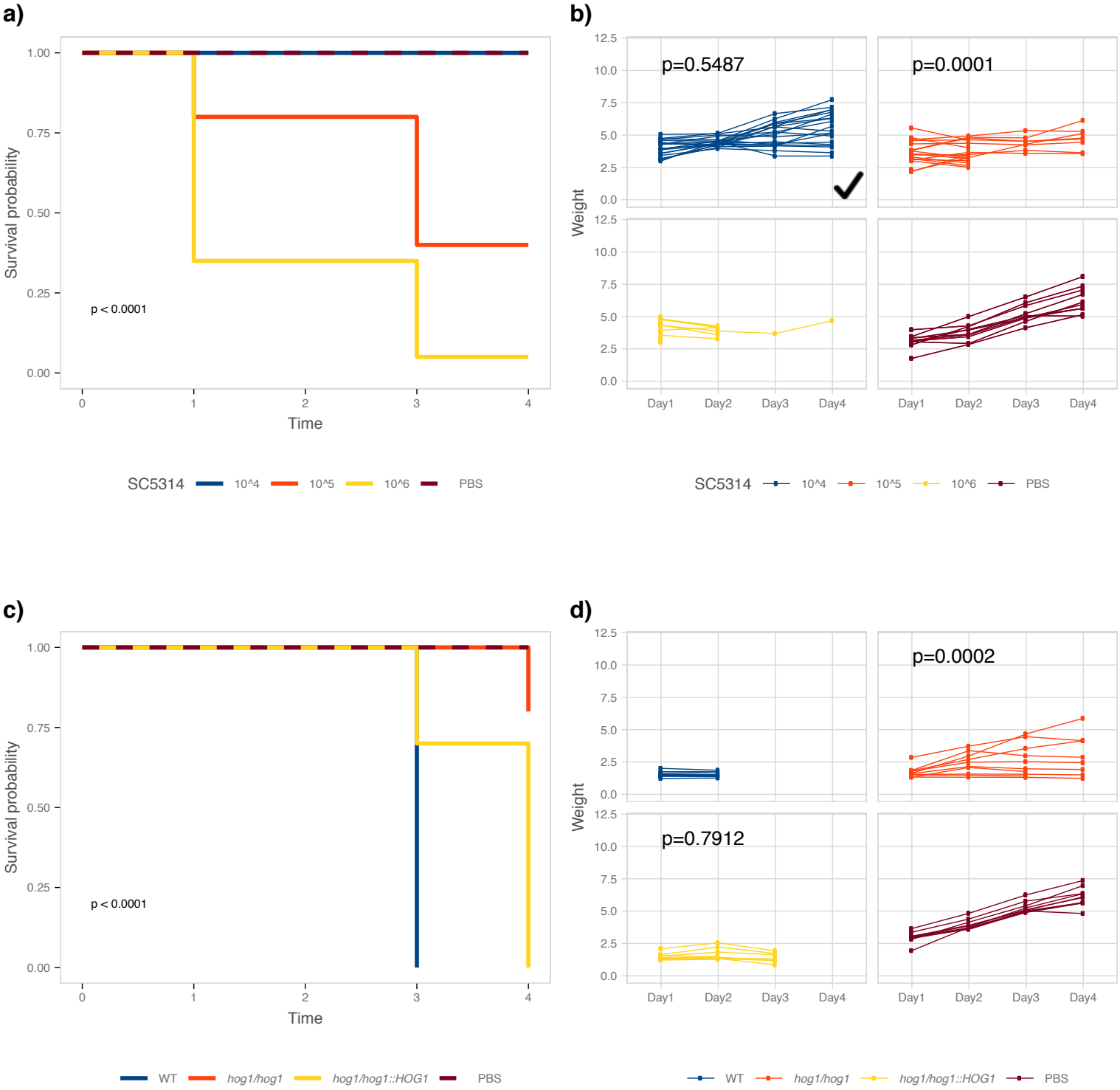


Figure 3 - Lyons et al.

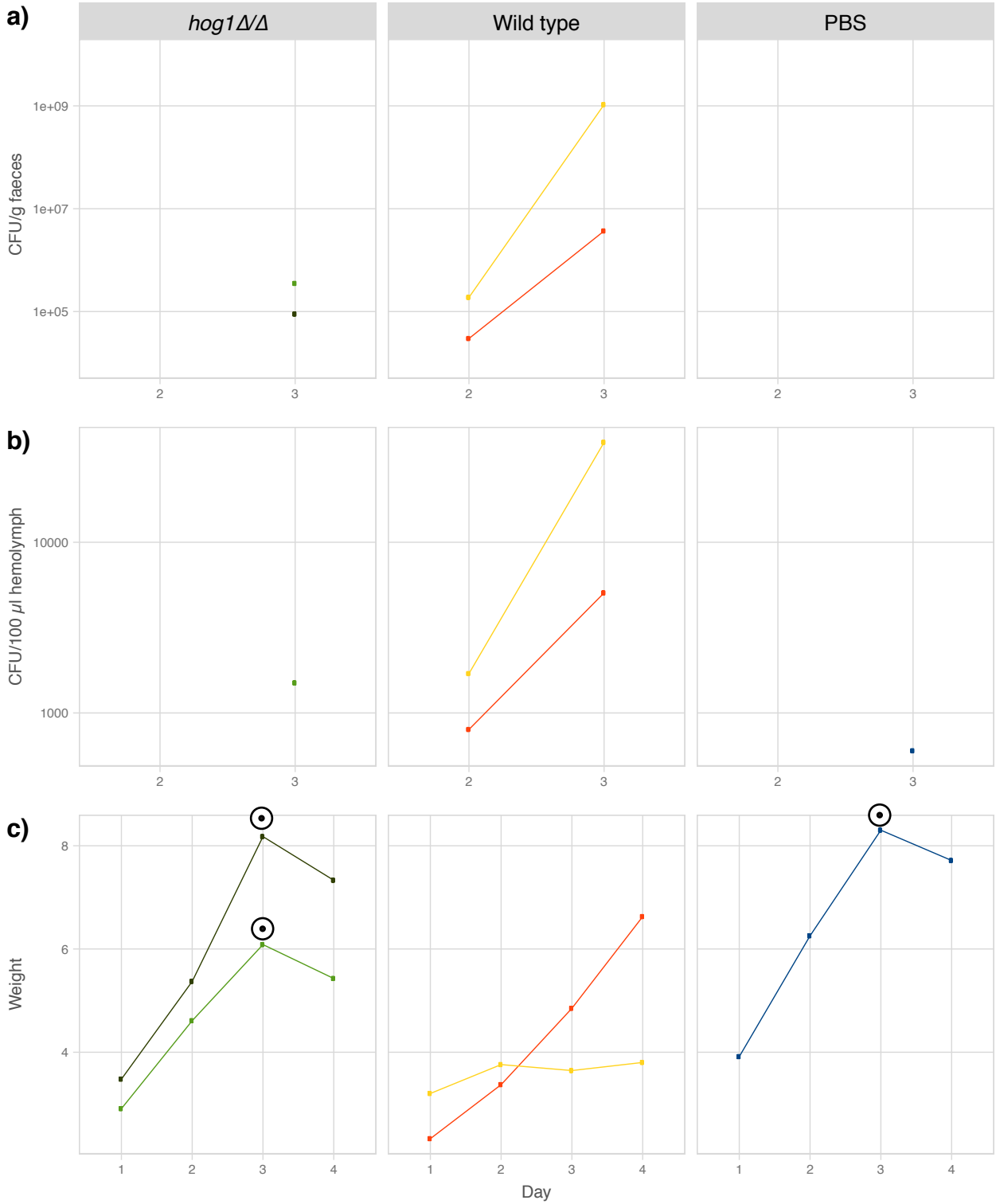


Figure 4 - Lyons et al.

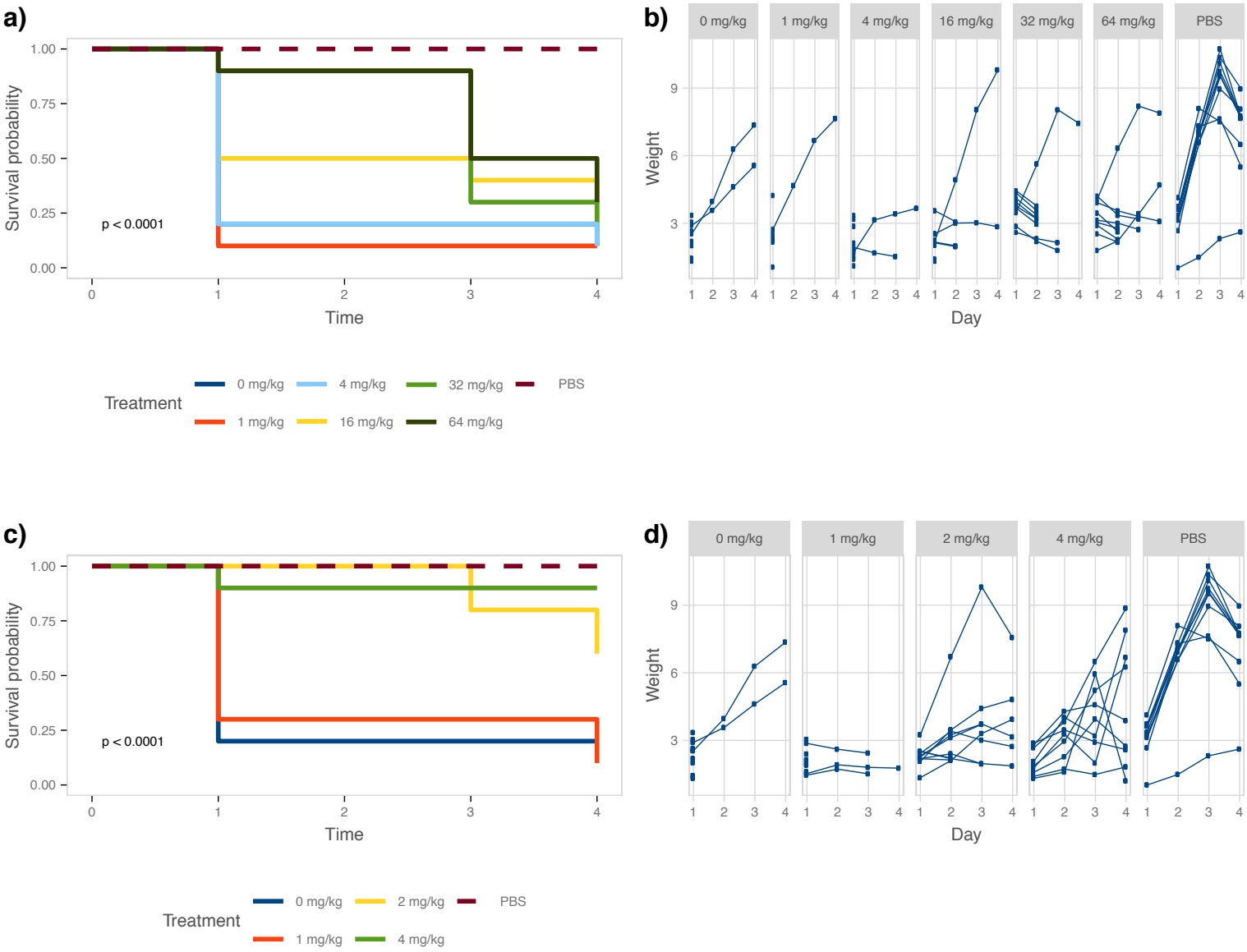


Figure 5 - Lyons et al.

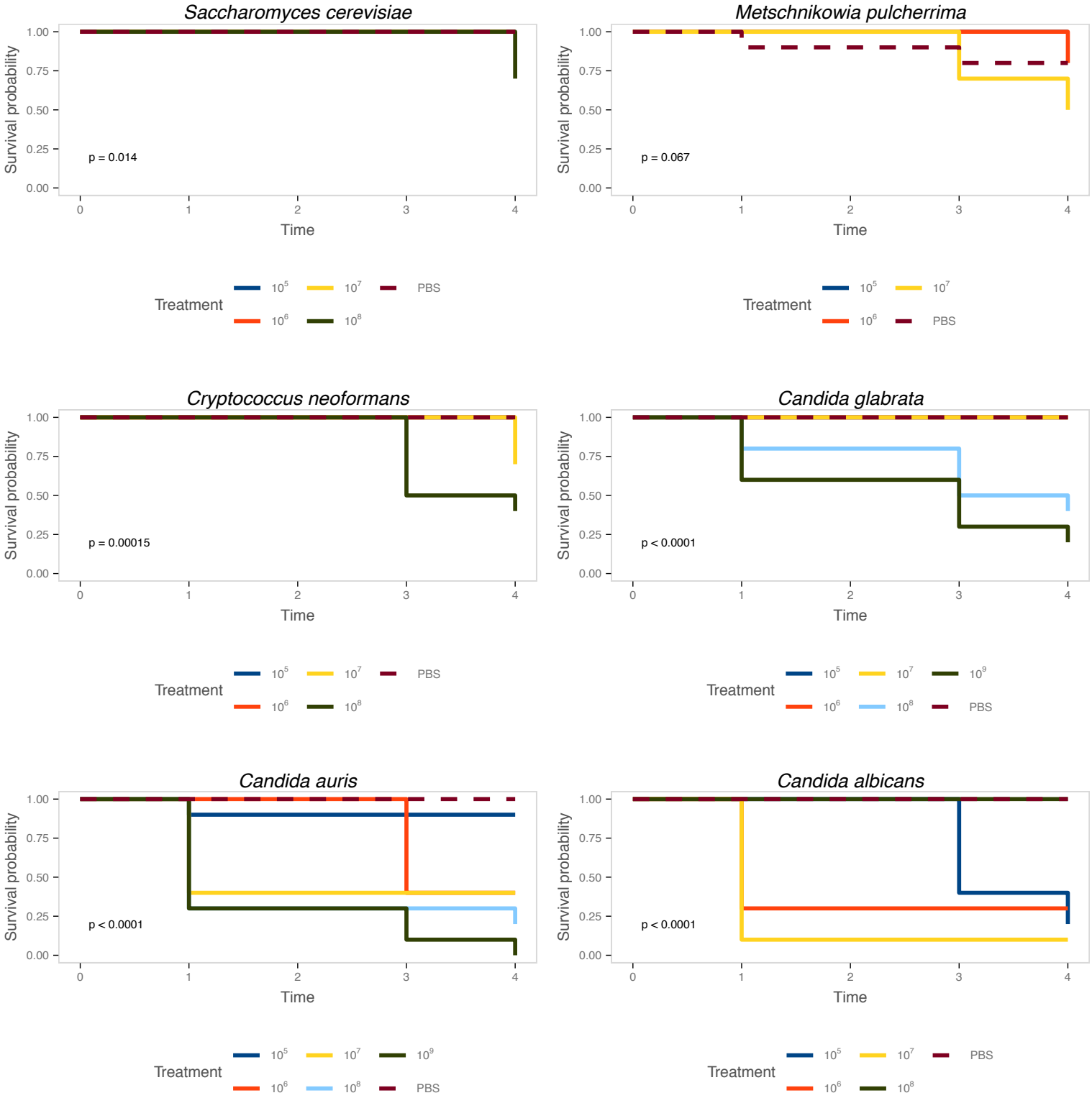


Figure 6 - Lyons et al.

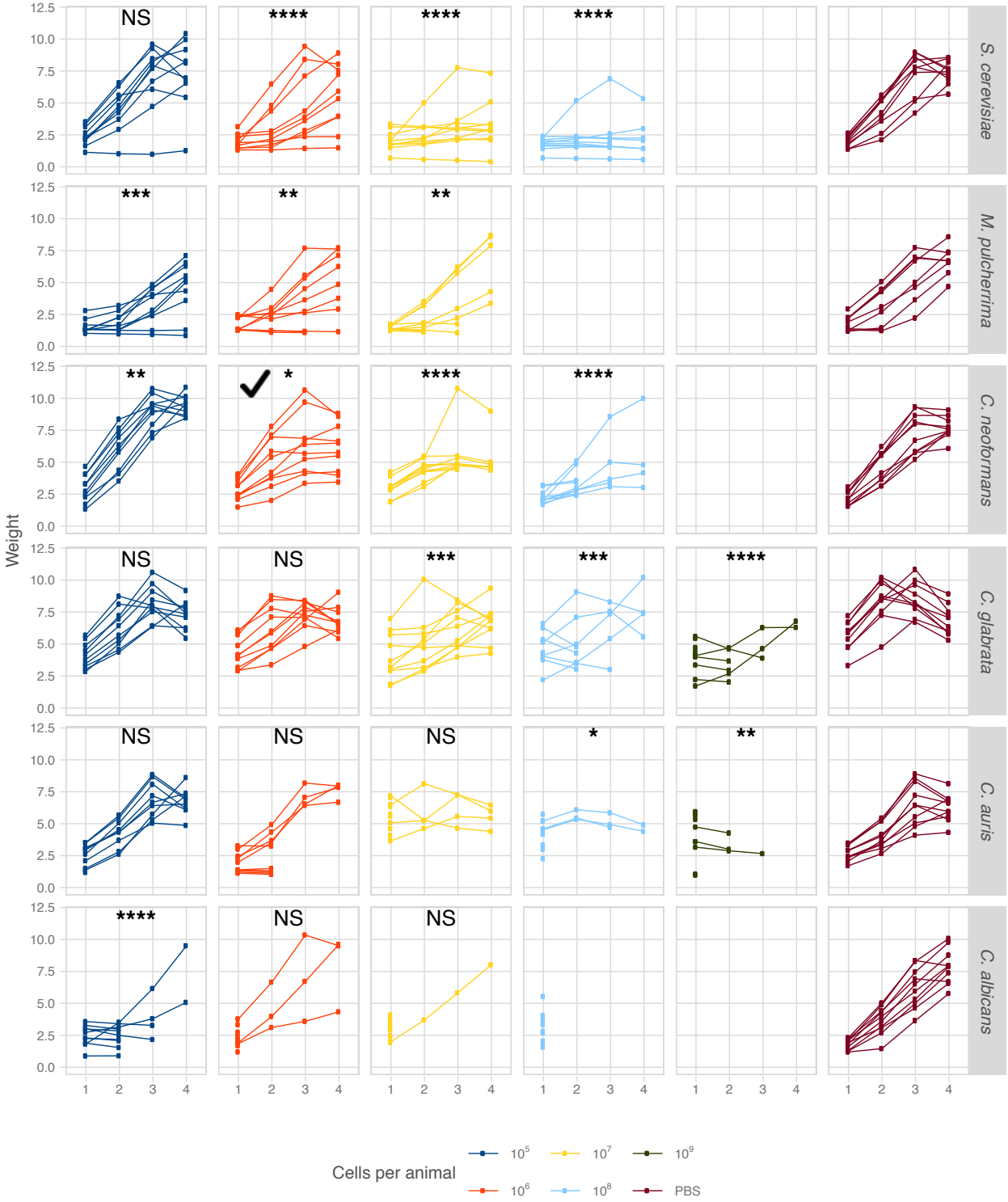
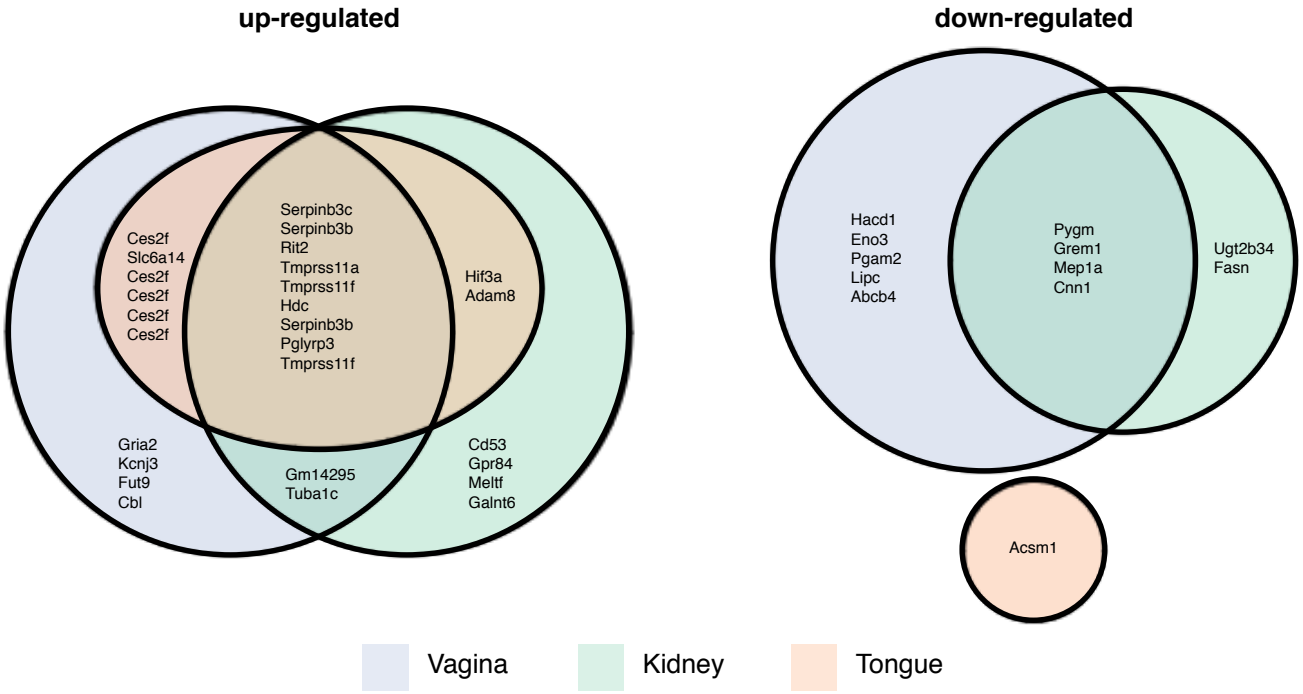


Figure 7- Lyons et al.



Supplemental Online Material

Figure S1 - Lyons et al.

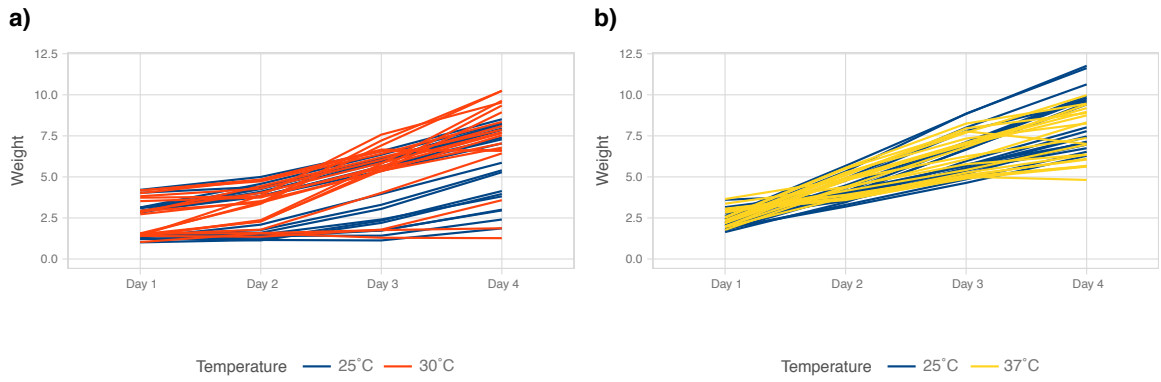


Figure S1: Caterpillar growth is comparable between standard colony temperature and host body temperature. Groups of ten animals were kept on standard diet at 25˚C and 30˚C **(a)** or 25˚C and 37˚C **(b)** for four days. Animals were scored daily for survival and weight as a measure of development. Within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

Figure S2 - Lyons et al.

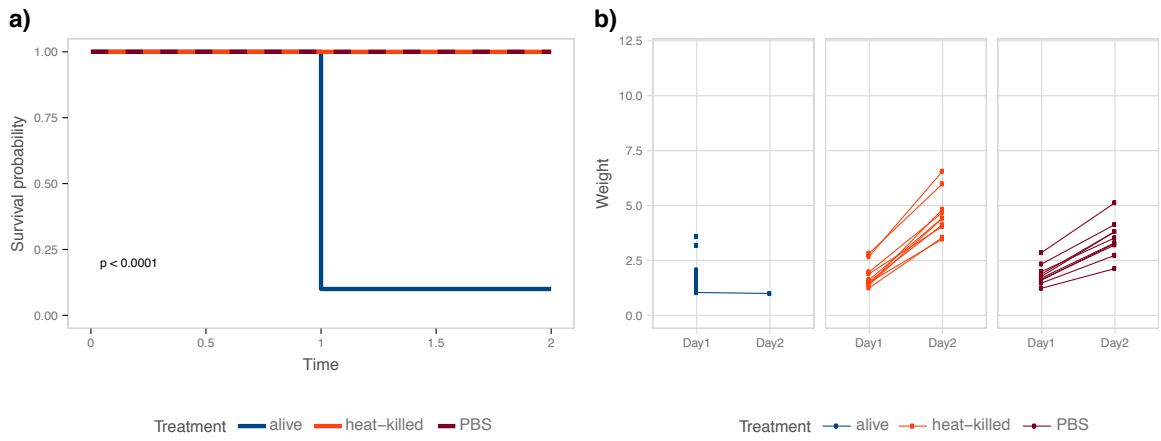


Figure S2: Heat-killed *C. albicans* cells do not affect caterpillar survival or weight gain. Groups of ten animals were injected with either live *C. albicans* wild-type YSD85 cells, heat-killed cells, or PBS. **a)** Only live yeast cells kill caterpillars. **b)** The weight gain in animals infected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

Figure S3 - Lyons et al.

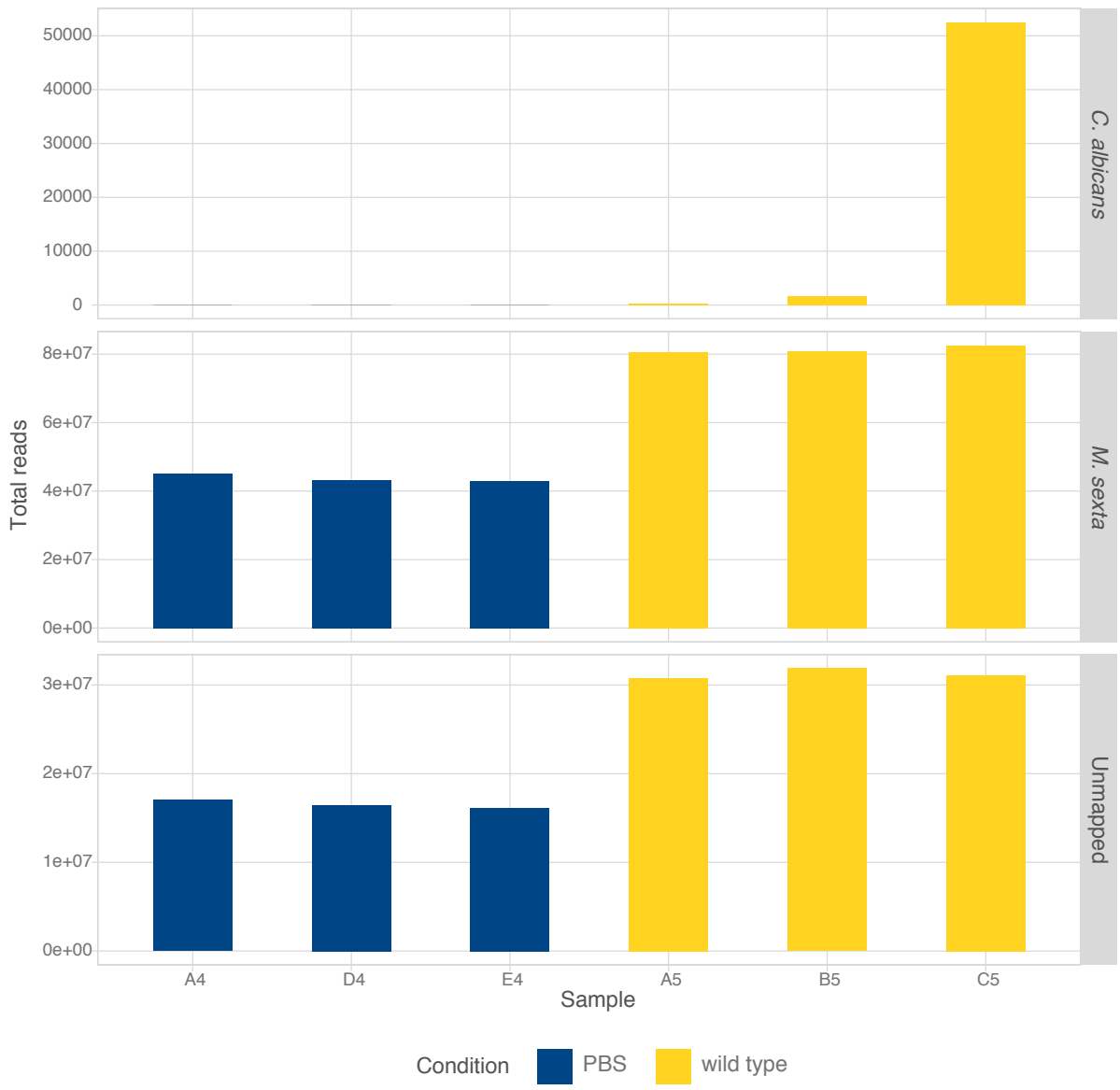


Figure S3: Mapping distribution of RNAseq reads. Between 17.8 M and 34.9 M read pairs per sample mapped either to the *M. sexta* or the *C. albicans*.

Figure S4 - Lyons et al.

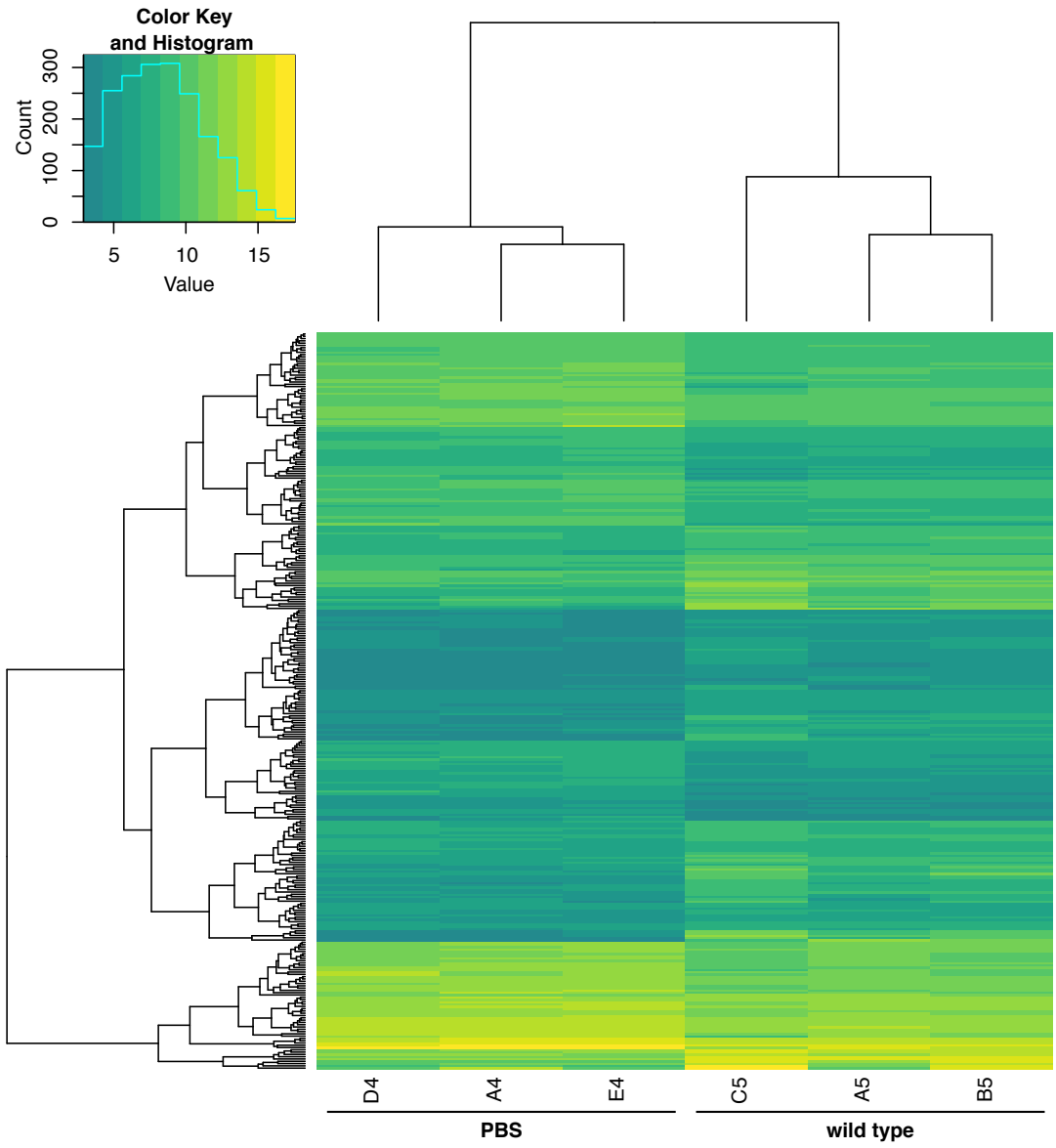


Figure S4: *M. sexta* genomic expression programs in response to infection with *C. albicans*. Hierarchical clustering of global gene expression patterns recorded in samples from uninfected control animals (PBS, samples A4, D4, E4) and those infected with the *C. albicans* wild type (samples A5, B5, C5). Listed are 322 differentially expressed genes on the y-axis in corresponding order to Table S1. In the inset, count refers to the number of differentially expressed genes and value denotes their magnitude of difference.